Investigations into possible interactions between Trichoderma harzianum genotypes and Agaricus bisporus

Williams, Josephine

Award date:
2000

Awarding institution:
University of Bath

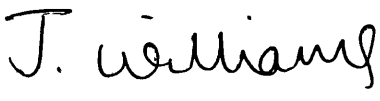
Investigations into Possible Interactions between *Trichoderma harzianum* genotypes and *Agaricus bisporus*

Submitted by Josephine Williams

for the degree of PhD

of the University of Bath

2000



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Trichoderma harzianum is a widely recognised biocontrol agent, capable of parasitism of several economically important phytopathogens. However, it is also responsible for the devastating green mould of the commercial mushroom, *Agaricus bisporus*. *T. harzianum* infestations, responsible for substantial financial losses to the industry, were first reported in 1985. Strains of *T. harzianum* capable of causing green mould have been classified as genotype 2 (Th2) in Europe and genotype 4 (Th4) in North America. Genotypes of *T. harzianum* associated with mushroom compost but with no effect on the growth of *A. bisporus* have been termed Th1 and Th3. The mechanism by which *T. harzianum* inhibits the growth of *A. bisporus* is unknown.

All genotypes of *T. harzianum* secreted depolymerases potentially capable of degradation of cell walls of *A. bisporus* and of wheat straw, the main component of mushroom compost. *T. harzianum* secreted enzymes in the order of protease and chitinase simultaneously, then laminarinase in the presence of *A. bisporus* cell walls as sole carbon source. Both chymoelastase and trypsin-like proteases (according to substrate specificities) were produced with an initial rapid increase for aggressive *T. harzianum* upon induction by *A. bisporus* cell walls. Isoelectric focussing of the proteins secreted on exposure to cell walls of *A. bisporus* or of wheat straw revealed many isoforms of chitinase, protease and endo-β-1,4-glucanase of which some were specific to aggressive *T. harzianum* strains. These included a protease isoform (pI 6.22), chitinase isoforms (pI 4.62 and 7.83) and endo-β-1,4-glucanase isoforms (pI 5.55 and 7.97). Genotypes were geographically defined by laminarinase isoforms secreted *in vitro*; European genotypes secreted uniform isozymes while Th4 produced unique isoforms (pI 6.19 and 6.25). Xylanase isoform profiles were consistent for all *T. harzianum* genotypes with the exception of Th3, which produced additional isoforms and this confirmed the recent re-classification of Th3 as *T. atroviride*. *T. harzianum* depolymerase activities were detected for the first time following axenic growth in sterile compost and showed substantial similarities with activities induced by wheat straw cell walls *in vitro*.

Interactions in tubes containing commercial compost confirmed a greater reduction of *A. bisporus* growth by aggressive than by non-aggressive *T. harzianum*. Models were

developed to investigate potential sources of *T. harzianum* contamination and to investigate and compare the aggressive behaviour of *T. harzianum* isolates. Scanning electron microscopy (SEM) of the interactions between *T. harzianum* and *A. bisporus* on nutrient agar media, spawn grain and in compost revealed infrequent evidence of mycoparasitism. Crystals rich in calcium were detected by SEM EDX in abundance on the mycelial surface of *A. bisporus*, which may have prevented contact with *T. harzianum*. Although secreted and cell wall-attached lectins were produced by Th1 and Th2, similar specificities suggested no link to "aggressiveness". Contrary to previous work, *T. harzianum* colonised compost in the absence of *A. bisporus* and the development of a selective medium enabled the quantification of *T. harzianum* growth in compost. This research contains the first known report of substantially greater (*ca.* 6.81-fold and 7.52-fold for Th2 and Th4 respectively) saprophytic growth in mushroom compost by aggressive *T. harzianum* than by non-aggressive *T. harzianum*. Therefore saprophytic colonisation of this substrate may be a prerequisite for Th2 and Th4 green mould infestations.

Development of a polymerase chain reaction (PCR) diagnostic test, based on previous work (HRI Wellesbourne) was attempted. Primers designed to the cellulase gene *cel1* of *A. bisporus* produced amplicons from *T. harzianum* genomic DNA. Aggressive *T. harzianum* DNA produced a 700 bp amplicon, while amplicons produced from non-aggressive DNA revealed variation. Amplicon sequences from Th2 and Th4 (but not Th1) were >90% homologous. Amplicons produced by *T. harzianum* shared no homology with *cel1* of *A. bisporus* suggesting random amplification of *T. harzianum* genomic DNA.

It is concluded that aggressive *T. harzianum* strains were combative, producing unique depolymerase isoforms and exhibiting a delayed sporulation strategy. Studies of interactions on nutrient agar and in compost suggested the occurrence of a competitive interaction when *T. harzianum* and *A. bisporus* were in close proximity. Effective scavenging of organic nutrients by *T. harzianum* as well as the production of cell wall-degrading enzymes and non-volatile inhibitory substances may, in combination, be primarily responsible for the inhibitory effect towards *A. bisporus*.

Acknowledgements

There are many people that I would like to thank in regards to the support, advice and encouragement that they have shown me throughout my PhD.

I would like to express my thanks to my supervisors Dr Richard Cooper, Dr John Clarkson and Dr Peter Mills for their support and guidance in all aspects of my research. In particular I would like to thank Dr Richard Cooper for the immense time and effort taken to edit my thesis and for the speed at which this was achieved.

I would also like to thank Chris Davey for his photography service, Dr Ursula Potter for her expertise in Electron Optics and her patience with my research samples, Chris Vennard for his technical support and Paul Christy for his invaluable advice about statistical analyses. The interest and support of Prof. David Wood and Dr Helen Grogan were also greatly appreciated.

I would especially like to thank the friends I have made during my PhD; in particular Dr Ben Cobb for all his support and advice throughout my research and the production of this thesis and Dr Amanda Carlile, who managed to survive working and living together for two years! I would also like to thank all those people I have worked with in the Microbial Pathogenicity Group at Bath University since 1995. I would also like to thank my friends; in particular, Dr Kim Taylor, who also managed to survive living together whilst we both prepared our PhD theses, Jane Wooler and Kelcey Roberts, and Chris Vennard for their continued encouragement and patience while I attempted to complete this period of my research.

And last but in no way least, special thanks to my family; thanks to my mother, Mary, my father, Richard and my brother, Russell. Their encouragement and never-ending support has been pivotal in all my achievements.

Abbreviations

bCA	Blended compost extract agar
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide
DWA	Distilled water agar
EDTA	ethylenediaminetetraacetic acid
EDX	Energy Dispersal X-ray microanalysis
fCA	Filtered compost extract agar
glc	glucose
glcNAC	N-acetylglucosamine
IAA	indole acetic acid
IEF	Isoelectric focussing
MEA	Malt extract agar
MES	2-(N-morpholino)ethanesulphonic acid
mQ	milli Q water
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
RAPD	random amplified polymorphic DNA
SN_L	signal to noise ratio
Tris	Tris(hydroxymethyl)aminomethane

Terminology used in previous reports is used throughout this study. Therefore when referring to the genotype (Th) the abbreviations in the "genotype" column will be used in the text and in parentheses after the strain.

T. harzianum *strain*	**Genotype**	**Action towards *A. bisporus***
Th1 (c)	Th1	Non-aggressive
TD15	Th1	Non-aggressive
T28JF	Th1	Non-aggressive
Th1M	Th1	Non-aggressive
TD7	Th3	Non-aggressive
IMI 110150	Th3	Non-aggressive
Th3c	Th3	Non-aggressive
A006022	Th3	Non-aggressive
T7	Th2	Aggressive
T32	Th2	Aggressive
Th3(05)	Th2	Aggressive
KPNT	Th2	Aggressive
Th2F	Th2	Aggressive
Th2A	Th2	Aggressive
RM10 casing	Th4	Aggressive
RM10 manure	Th4	Aggressive
BE	Th4	Aggressive

Chapter One

General Introduction

Aggressive strains of *T. harzianum* were responsible for crop losses of *A. bisporus* estimated at £3-5 million in the UK during the period 1987-90 and appear to be an ongoing problem to the industry (Seaby 1996b). These devastating *T. harzianum* green mould infestations often resulted in total loss mushroom crops. Similar outbreaks have been reported in North America, Canada and Australia. The strains responsible for the aggressive colonisation of mushroom compost are geographically distinct and have been classified as genotype 2 (Th2 - UK and Ireland) and genotype 4 (Th4 - North America and Canada) (Muthumeenakshi *et al.* 1998). The difference has been revealed as a five base pair difference in the rDNA internal transcribed spacer (ITS) 1 between Th2 and Th4 (Muthumeenakshi *et al.* 1994, 1998). Other *T. harzianum* genotypes found in association with mushroom compost developed localised colonisation with no effect on the growth or mushroom yields of *A. bisporus*. These non-aggressive compost colonising strains have been assigned to Th1 and Th3 (Muthumeenakshi *et al.* 1994).

Manifestation of *T. harzianum* aggressive colonisation was observed as large areas of green conidia on the compost surface. Large numbers of the red pepper mite, *Pygmephorus mesembrinae* were attracted by *T. harzianum* conidia from which they fed and this further reduced mushroom yields (Seaby 1987; Staunton 1987). Poor hygiene awareness was suggested to be the basis for many of the *T. harzianum* green mould infestations; in addition, animal vectors and contaminated spawn were revealed (Seaby 1987; Staunton 1987).

Previous research has focused on the epidemiology of *T. harzianum* green mould infestations, possible infection routes and disease development. Another aspect of research was to find a rapid control measure for growers with persistent *T. harzianum* infections. A selective fungicide Bavistin DF has been shown to provide protection of *A. bisporus* mycelium during establishment from spawn (Fletcher and Young 1995).

While improved hygiene regimes have significantly reduced the level of incidents, the mechanisms of antagonism employed by aggressive genotypes of *T. harzianum* are not

well understood. *T. harzianum* is an antagonist of several plant pathogenic fungi and as such has been considered as a potential biocontrol agent. In most systems studied, *T. harzianum* was mycoparasitic, growing towards and coiling around the host (Elad *et al.* 1983b) as well as producing host cell wall depolymerases during infection (Elad *et al.* 1982). The objectives of this study were to elucidate the mechanism of antagonism used by aggressive *T. harzianum* genotypes.

The aim of this research was to focus on the differences between aggressive and non-aggressive genotypes of *T. harzianum* and to determine genotype-distinguishing characteristics at ultrastructural, biochemical and molecular levels. Ultrastructural studies were made of *T. harzianum* and *A. bisporus* interactions on various substrates including: nutrient agar, compost and spawn grain. The depolymerase activities of *T. harzianum* genotypes were characterised and in depth studies revealed vital differences at the isozyme level. The polymerase chain reaction (PCR) was used as a basis for a potential diagnostic test for the presence of aggressive *T. harzianum* strains.

Literature Review

1.0 Production of the commercial mushroom – *Agaricus bisporus*

Cultivation of the mushroom, *Agaricus bisporus*, was introduced in the late seventeenth century. However, the preparation of a specific substrate for the process was not described until a century later by Abercrombie (1779). Global cultivation of mushrooms increased rapidly and now mushrooms are the single highest value crop of all fruit and vegetables in the UK. The UK alone produced 118000 tonnes of mushrooms fetching *ca.* £165M at farm gate prices (MAFF 1994). The value of the global *A. bisporus* industry is *ca.* £2500M (Elliot, 1997). Protein accounts for approximately 30% of the dry matter (variation between crops and species) and essential minerals and vitamins are also provided (Elliot 1997). This section will cover the cultivation of *A.bisporus* and the preparation of mushroom compost.

1.0.1 Preparation of compost and the involvement of the microflora

A. bisporus is a heterotroph and acquires carbon in the form of complex compounds such as plant polysaccharides. Compost provides the nutritional requirements of *A. bisporus* in an environment that is semi-selective to the fungus. The physical qualities of compost including free permeability towards air, water holding capabilities and appropriate pH are all suitable for the cultivation of *A.bisporus* (Fermor *et al.* 1985). The chemical state of compost, i.e. the carbohydrate and nitrogen sources, vitamins and minerals provide an environment in which *A. bisporus* has the competitive edge over other micro-organisms. Carbohydrates are present as cellulose, hemicellulose and lignin, while nitrogen is also obtained from the nitrogen-rich lignin-humus complex (Gerrits 1988). Few fungi are capable of obtaining carbon and nitrogen from lignin and therefore by producing laccases, cellulases and hemicellulases *A. bisporus* succeeds in a selective environment. *A. bisporus* appears to have a symbiotic relationship with the microflora present in compost; other fungi may not have this tolerance (Gerrits 1988).

In the UK, the principal ingredient of mushroom compost is wheat straw supplemented with poultry manure. The relationship between composts generated from waste and mushroom growing has influenced much research into compost composition (Poppe and Hofte 1995). Waste products can be used directly as a substrate, e.g. cereal straws, or as additives to poor substrates, e.g. poultry manure (Poppe and Hofte 1995).

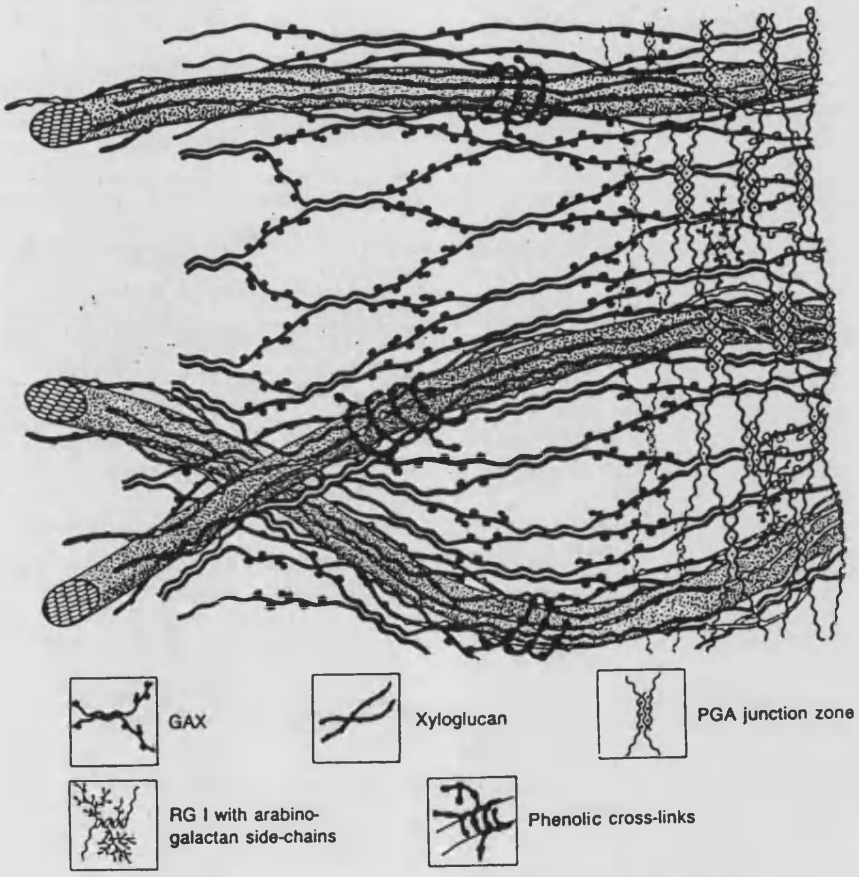
The straw component gives compost the desired density and structure; thin-walled straws with hollow centres make the best compost (Tunney 1977; Fermor *et al.* 1985). During the composting process, up to 40% of the dry matter is lost due to the degradative action of the microbial biomass. This microflora attacks the straw, changing its chemical and physical structure.

Primary cell walls of wheat

Primary cell walls of plants have been reviewed in detail by McNeil *et al.* (1984), Carpita and Gibeaut (1993) and Carpita (1996); it is from these papers that the following description of wheat straw walls is composed. They are detailed here as they represent a potential substrate for cell wall-degrading enzymes of *T. harzianum* and *A. bisporus*. Primary cell walls of wheat are classified as type II plant cell walls (Fig 1.1). Type II cell walls consist of cellulosic microfibrils interlocked with polymers of glucuronoarabinoxylans (GAXs). Several chains of cellulose (polymers of (1→4)β-linked D-glucose) are arranged in parallel to form microfibrils. GAX is the matrix component of the plant cell wall, reinforcing the structure and strength of the microfibrils. GAX comprises linear chains of (1→4)β-linked D-xylose to which side chains, typically arabinose and glucosyluronic acid (GlcA) may attach. The extent of this substitution affects the ability to bind to other GAX and cellulose; an unbranched polymer of (1→4)-linked xylan is capable of binding to cellulose or another GAX via hydrogen bonds. Side chains interfere with hydrogen bonding preventing binding between GAX and cellulose. Hence the presence of highly branched GAXs in the dividing and elongating stages of plant cell growth.

Type II cell walls contain small amounts of xyloglucan, compared to type I walls, and have very low pectin content. The pectins present in type II cell walls are polygalacturonic acid (PGA), helical homopolymers of (1→4)α-D-galacturonic acid and rhamnogalacturonan I (RG I) and heteropolymers of repeating (1→2)-α-L-rhamnosyl-(1→4)-α-D-galacturonic acid disaccharides. Pectins are associated with highly substituted GAX (HS GAX) and may have a role in controlling wall metabolism and growth. Finally, the cleavage of side chains attached to HS-GAX could result in stabilising the wall growth by allowing binding to occur between GAX to itself and cellulose, locking the wall into a rigid structure.

Figure 1.1. The type II cell wall of the Poaceae (Reproduced from Carpita and Gibeaut 1993)



Representation of a single stratum of microfibrils just after cell division. The microfibrils are interlocked by glucuronoarabinoxylans instead of xyloglucans. The xylans are probably highly substituted and the units are cleaved in the extracellular space to yield stretches of xylan that can bind to either cellulose or each other (Carpita and Gibeaut 1993).

Primary cell walls also contain structural and enzymatic proteins and glycoproteins. Structural proteins stabilise cell shape by covalent cross-linkage of the primary and secondary cell walls. There are several structural proteins in type II cell walls and these include threonine-hydroxyproline-rich glycoprotein (THRGP). In some type II cell walls glycine-rich proteins (GRPs) are found which have cytosolic and cell wall functions. Arabinogalactan proteins (AGPs) are glycoproteins, which are a major component of secretory vesicles. These glycoproteins could have a role in transporting cell wall components to the expanding cell wall, where they too are incorporated. The enzymatic proteins in cell walls include hydrolases, transferases, peroxidases, phosphatases and esterases. Such enzymes permit the modification of the chemical composition of the cell wall that results in cell wall growth and the interlocking of a rigid structure. In addition, enzymes with activity towards pathogens are also present within the primary cell wall.

Cross-linking of GAX is achieved via esterified and etherified hydroxycinnamates and other phenolic substances. Such linkages are responsible for the attachment of lignin to GAX during secondary cell wall formation. Lignin is a polymer of phenylpropanoid units (coniferyl, sinapyl and p-hydroxycinnamyl alcohols). Phenolic acids are bifunctional and can bind through ester linkages (*via* the carboxyl group) or by ether linkages (*via* the hydroxyl group) (Scalbert *et al.* 1985). This recalcitrant polymer provides additional strength to the cell wall and shields structural polysaccharides from microbial attack due to the following properties: large size of polymer, high degree of branching, diversity of monomers, diversity of linkages and stability of C-C and ether bonds.

A succession of microorganisms during fermentation results in the preparation of a selective compost. The microflora functions to remove readily available carbon and nitrogen sources thus depriving weed moulds of nutrients. Another element of selectivity is the production of a protein-lignin complex that *A. bisporus* alone is capable of metabolising (Gerrits 1969).

Micro-organisms are introduced to the compost with the raw ingredients. Wheat straw harbours 3 groups of fungi: i) primary saprophytes e.g. *Cladosporium herbarum*, ii) fungi often found on stored materials e.g. *Aspergillus* spp. and *Penicillium* spp. and iii) opportunistic phycomycete 'sugar-fungi' e.g. *Mucor hiemalis* (Chang and Hudson 1967). A large bacterial population (>8 morphologically distinct rod and spherical bacteria) was

associated with stable manure (Atkey and Wood 1983). A large proportion of the microflora was found in the phloem but distribution was generally widespread (Atkey and Wood 1983). Composting consists of two phases and a succession of microbes flourish under the varying conditions.

At phase I the raw ingredients are pre-wetted and compost stack self-heats in an uncontrolled manner. Initially mesophilic microbial (bacteria, actinomycetes and fungi) populations develop and utilise readily available nutrients since these microbes do not possess cellulolytic activity (Fermor *et al.* 1979, 1985; Gerrits 1988). Decomposition of proteins and the release of ammonia increases the compost temperature and also softens the straw. Increased temperature results in the decline of mesophilic population and succession of the thermophiles; this 'establishment' stage typically lasts for 3 days (Hayes 1977; Atkey and Wood 1983). Temperature of the compost continues to rise and thermophilic bacteria become dominant (Atkey and Wood 1983; Evered *et al.* 1995). Regular turning and mixing of the compost stack eliminates the development of an anaerobic core.

Phase II of composting is divided into two processes, both controlled by air temperature. Pasteurisation (stage 1) is the controlled heating of the compost and this stage attempts to destroy all potential pests and pathogens. During this stage thermophilic bacterial populations degrade the epidermis and middle lamellae of straw cells (Atkey and Wood 1983). Bacteria also degrade the primary walls of the xylem vessels and reveal the annular rings of secondary supporting structures (Atkey and Wood 1983).

A decrease in temperature to 45-50°C marks the beginning of 'conditioning' (stage 2), which is optimal for the growth of thermophilic actinomycetes and fungi (Fergus 1964). Populations of thermophilic actinomycetes and fungi increase and eventually dominate the microflora. The cellulose and hemicellulose contents are broken down, while the lignin-humus content remains the same (Gerrits 1988). Sources of ammonia, inhibitory towards *A. bisporus* growth, are removed by incorporation into microbial biomass and in the flow of air throughout the compost stack. Fungi and actinomycetes on the surface and within straw cells are responsible for the complete erosion of large areas of the epidermis thereby exposing other cells (Atkey and Wood 1983).

The regeneration of mesophilic fungi at the end of the fungal succession indicates a decrease in core temperature. The 'spawning' procedure occurs after a further drop in temperature (*ca.* 25°C). At the optimal temperature for *A. bisporus* growth, thermophiles are fungistatic and mesophiles are unable to obtain readily available nutrients therefore *A. bisporus* can outcompete other rivals. *A. bisporus* is added in such large quantities that mesophiles pose little threat of competition (Gerrits 1988).

1.0.2 Factors influencing the composting process

It is estimated that fruiting bodies obtain 70-90% of water intake from the compost (Gerrits 1988). The optimum moisture content of synthetic compost at spawning is 69% and this falls to 66% after mycelial growth of *A. bisporus* (Gerrits 1988). Water content has important implications during fructification; lowering the water potential of compost results in mushrooms with higher dry matter content, however this is offset by a reduction in yield (Gerrits *et al.* 1995).

The heating capacity of the compost is dependent on the concentration of ammonium (Gerrits 1988). Chicken manure is frequently used as a nitrogen supplement (Gerrits 1977). Ammonia is a readily available source of nitrogen used by microbes since nitrogen complexed in proteins is inaccessible to microbes (Gerrits 1977). Optimal mushroom yields are achieved at an ammonium content of 0.4% (higher levels are inhibitory to *A. bisporus*) at 'filling' (beginning of phase II) and a constant nitrogen content at 2.0% (Gerrits 1977). Optimal fruiting body formation and yields require C:N ratio of 17C:1N (Hayes 1977).

Regular turning and mixing of the stack during phase I ensures aerobic fermentation (Tunney 1977). The addition of gypsum flocculates colloids, increasing the porosity and texture of compost to enable the flow of air through the compost stack (Pizer and Thompson 1938). The airflow also controls the compost temperature and removes waste gases such as carbon dioxide, water vapour and excess ammonia (Randle 1977).

1.0.3 The role of the microflora during A.bisporus colonisation of compost

Populations of actinomycetes and fungi remain abundant during 'spawning' (Atkey and Wood 1983). *A. bisporus* may use actinomycetes as a nutrient source as they are often found in association (Atkey and Wood 1983). The thermophilic fungus, *Scytalidium*

thermophilum has been implicated to stimulate *A. bisporus* growth since there is a positive correlation between density of *S. thermophilum* and mushroom yields (Straatsma *et al.* 1994, 1995). *A. bisporus* rapidly colonises the straw surfaces and crystals of calcium oxalate cover the hyphae (Atkey and Wood 1983). In contrast, hyphae of *A. bisporus* within straw cells do not produce calcium oxalate crystals. *A. bisporus* failed to produce fruiting bodies in sterile casing, but the addition of non-sterile material resulted in fructification (reviewed by Gerrits 1988). Pseudomonads are common in casing soil and have often been observed in association with reproductive hyphae of *A. bisporus* within the casing soil (Masaphy 1987). Calcium oxalate crystals are present on vegetative mycelium but not on reproductive hyphae of *A. bisporus* and the crystals were thought to prevent fructification by interfering with strand formation (Masaphy *et al.* 1987). Interestingly, Pseudomonads were thought to be capable of utilising calcium oxalate under the conditions present in the casing (Masaphy *et al.* 1987).

On completion of the composting process, compost is cooled to *ca.* 24°C prior to the inoculation of *A. bisporus*, or 'spawning'. Spawn consists of cereal grain colonised by *A. bisporus* mycelium, which is mixed into the compost and allowed to colonise at 24°C with high relative humidity and *ca.* 2% carbon dioxide. This process is complete within 10 to 14 days (Gaze 1985). Fructification is induced by the addition of an inert material 'casing soil', prepared from sphagnum peat and limestone which is of neutral to alkaline pH (Gaze 1985; van Gils 1988; Fletcher *et al.* 1989). Casing provides anchorage for the developing sporophores and acts as a reservoir for water, essential for high yields (Fletcher *et al.* 1989; Visscher 1988). This layer also regulates the evapouration of water.

Mycelial establishment in the casing layer occurs at 20°C and the temperature is subsequently lowered to 16 to 20°C at fruit body initiation (Gaze 1989). Typical incubation periods of 18 to 21 days are required from casing to the harvest of the first flush. Subsequent flushes are harvested in the following 4 to 5 weeks (Gerrits 1988; Fletcher *et al.* 1989).

Techniques to improve quality and increase yields include supplementation and 'ruffling' of the casing. Supplements can increase yields by up to 20% (Fletcher *et al.* 1989). Timing of supplement application is very important since supplementation in the presence of

"weed moulds" provides readily available sources of carbon and nitrogen and can exacerbate weed mould problems (reviewed in the following: Fermor *et al.* 1985; Gerrits 1988; Fletcher *et al.* 1989). Addition of supplements during later stages such as casing is thought to lower the risk of weed moulds. Ruffling, the disturbance of the colonised casing layer, results in increased yields and more uniform appearance of pinheads in terms of size and time of emergence (Visscher 1988; Fletcher *et al.* 1989). Deep ruffling dramatically increased mushroom yields and was suggested to allow the diffusion of CO_2 out of and O_2 into the compost (Visscher 1988), however it may exacerbate localised infections (Fletcher *et al.* 1989).

1.0.4 Stages of mushroom development in the cultivation of A. bisporus

A. bisporus is a member of the Basidiomycotina. The vegetative mycelium possesses distinctive dolipore septa. Fruiting bodies develop from the vegetative mycelium by aggregation to form strands or rhizomorphs (Townsend 1954). Rhizomorphs comprise 2 layers; an inner core of tightly packed, often highly vacuolated hyphae and an outer layer of narrow, randomly arranged hyphae (Wood *et al.* 1985). The primordium is the earliest stage of the fruiting body and is 1-2 mm in size (Wood *et al.* 1985). The developing fruiting body differentiates into the cap and stipe. The gill tissue of the cap matures in the annular zone, which initially develops as two cavities (Wood *et al.* 1985). From gill tissue basidiospores are produced. Three regions constitute the gill tissue and these are: the trama, the sub-hymenium and the hymenium. The highly vacuolated cells of the trama are attached to the cap and run longitudinally down the centre of the gill (Wood *et al.* 1985). The sub-hymenial cells are branched cells, which develop from the trama at intervals. Complex branching and hyphal growth from the sub-hymenial layer produces the hymenium layer, which consists of closely packed basidial cells (Wood *et al.* 1985; Flegg and Wood 1985). A swelling develops from the basidial cell, termed a sterigma. The tip of the sterigma continues to enlarge eventually forming a basidiospore. Approximately 4000 basidia are formed per mm^2 of gill tissue (Flegg and Wood 1985).

The stipe functions to elevate the cap from the casing surface, aiding dispersal of the basidiospores. The stipe consists of the inner core surrounded by the outer stipe (Wood *et al.* 1985). At the base of the stipe thin, closely packed hyphae can be observed to mass irregularly. The mid- and upper regions of the inner core consist of irregular arrangements

of hyphae, less tightly packed and in vertical orientation. A layer of long slender hyphae in horizontal orientation can be found on the outer stipe (Wood *et al.* 1985).

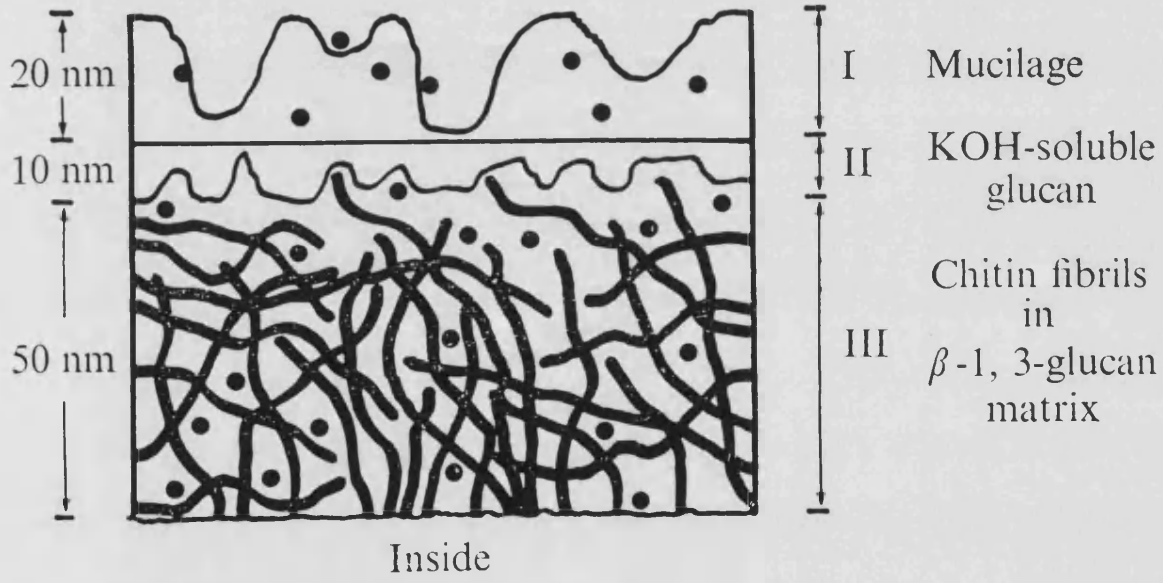
The cap continues to enlarge from the primordial (pinhead) stage and the mature fruiting body can be harvested according to the desired size cap. Mushrooms in early stages of development have an intact veil protecting the basidia on the gills. In fully matured mushrooms the veil tears, gills are exposed and the basidiospores are dispersed.

The cell walls of *A.bisporus* are detailed here, as they will be considered later as a potential substrate for *T. harzianum* cell wall-degrading enzymes. The cell walls can be described as glucan-chitin due to the major, constitutive polysaccharides (Bartnicki-Garcia 1968). Proteins and lipids are also present in lesser quantities. The cell wall is complex and this has been demonstrated by degradation with a variety of enzymes and acid and alkali-extractions (Michalenko *et al.* 1976). Architecture of the cell wall has been studied by Michalenko *et al.* (1976) and consists of an inner layer of chitin microfibrils in a β-glucan and protein matrix and an outer layer of alkali-soluble α-glucan (Fig 1.2). The innermost layer of the chitin-glucan layer is thought to be compact and even, while the outer part is probably uneven and loose to allow attachment and integration with the alkali-soluble α-glucan layer. The external surface could be coated in β-glucan mucilage, which also contains protein. Protein is found throughout the wall and there are no discrete layers of individual wall polymers, hence the requirement for several degradative enzymes.

Differences in the cell wall chemical composition of *A. bisporus* mycelium and fruiting bodies have been reported (Novaes-Ledieu and Garcia Mendoza 1981; Garcia Mendoza et al 1987). The vegetative and reproductive stages of *A. bisporus* differ in the proportions of the components and some of the linkages in the corresponding cell walls.

The carbohydrates (*ca.* 84% in total) found in mycelial cell walls are 43% (w/w) chitin, 14% alkali-soluble α-glucan and 27% β-glucan (Michalenko *et al.* 1976). Fruiting body cell walls contained less total carbohydrate (*ca.* 78%) than mycelial cell walls however, significantly more lipid was present (*ca.* 10% fruit body and 1.5% mycelial) (Novaes-Ledieu *et al.* 1981). Fatty acids have important roles in the fruiting body, thus providing an explanation for the increase in lipid content of fruiting bodies (Cruz *et al.* 1997). The major

Figure 1.2. Diagrammatic model of *A.bisporus* cell wall (Reproduced from Michalenko *et al.* 1976).



• indicates protein.

fatty acid is linoleic acid (18:2), the precursor of mushroom alcohol, 1-octen-3-ol (Tressl *et al.* 1982). This alcohol and two ketones, 1-octen-3-one and 3-octanone, are largely responsible for mushroom flavour (Cronin and Ward 1971). In addition, mycelial cell walls contained more protein than did the cell walls of fruit bodies; typically *ca.* 12.5% and 9.5% for mycelial and fruit body cell walls respectively (Michalenko *et al.* 1976; Novaes-Ledieu *et al.* 1981).

The other major difference between vegetative and reproductive hyphae is the nature of glycosidic bonds in the cell wall polysaccharides. Vegetative hyphal cell walls contain high amounts of (1→3) and few (1→6) linkages, while cell walls of reproductive hyphae are rich in (1→6) linkages (Garcia Mendoza et al 1987; Mol and Wessels 1990). The presence of (1→6) side chains may prevent the organisation of (1→3)-β-glucan chains into regular packing and hence allow expansion of whole cell wall (Mol and Wessels 1990). The reproductive hyphal cell walls are more sensitive to chitinase activity and this reflects the less organised structure of these cell walls that allow for diffuse expansion (Mol and Wessels 1990). The vegetative hyphae grow by apical extension, while cells of the fruiting body grow via diffuse extension, which occurs over the whole wall surface (Craig *et al.* 1977).

1.0.5 Nutrition of A. bisporus

A. bisporus requires carbon and nitrogen compounds, essential elements and vitamins and growth factors (Spencer and Wood 1985). *A. bisporus* preferentially hydrolyses the ligno-protein complex during the vegetative phase of colonisation (Wood and Goodenough 1977; Turner 1977). This degradation can be determined by following the laccase activity of *A. bisporus*, which increases throughout vegetative growth and rapidly decreases at the onset of fruiting (Wood and Goodenough 1977; Turner 1977). Laccases are copper bearing oxidases capable of catalyzing one electron oxidation of the phenolic moieties of lignin, which release reactive phenoxy radicals (Sinsabaugh and Liptak 1997). Ligno-protein complexes are probably the major source of nitrogen during the vegetative phase of growth (Wood and Goodenough 1977). Cellulase activity of *A. bisporus* is relatively low until an increase at the onset of reproduction (Turner *et al.* 1975). High metabolism necessary to produce sporophores and therefore cellulase activity is more important during fructification (Wood and Goodenough 1977). This activity is cyclical since the elevated activity of

cellulase dropped subsequent to harvest and increased again with the development of the next flush suggesting a role in regulation (Claydon *et al.* 1988). Hemicellulases are also produced by *A. bisporus* allowing the hydrolysis of more accessible sources of carbon. The activity of laminarinase and xylanase produced by *A. bisporus* is not linked to any particular growth stages (Wood and Goodenough 1977).

The microflora also represent a source of nutrients that comprises *ca.* 2% of total compost dry weight (Wood and Fermor 1985). The microflora provides the only supply of fatty acids and lipids (Fermor and Grant 1985). To access these nutrients *A. bisporus* must degrade microbial cell wall polymers.

Cell walls of bacteria are largely peptidoglycan (Pelczar *et al.* 1986). Peptidoglycan differs in composition according to species but basic units include, *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-alanine, D-glutamate and a diaminoacid (LL- or meso-diaminopimelic acid, L-lysine, L-ornithine or L-diaminobutyric acid). Gram positive bacteria, which differ to Gram negative, contain a high proportion of peptidoglycan (50% dry weight) and very little lipid. Gram-positive bacterial cell walls also contain teichoic acids; acidic polymers of ribitol phosphate or glycerol phosphate which covalently bond to peptidoglycan. The organisation of polymers in Gram-positive bacterial cell walls is relatively simple and appears homogeneous (Pelczar *et al.* 1986). Cell walls of Gram-negative bacteria are more complex and consist of several layers. Peptidoglycan represents just 10% of cell wall dry weight in Gram negative bacteria (Pelczar *et al.* 1986). An outer membrane rich in lipids (11-22% dry weight) surrounds the thin layer of peptidoglycan. The periplasmic space of 5-7nm separates the peptidoglycan and outer. The outer membrane renders Gram-negative bacteria relatively resistant to lysozyme, a peptidoglycan depolymerase (Pelczar *et al.* 1986). The outer membrane is a bilayer of phospholipids, proteins and lipopolysaccharide. Proteins link the outer membrane to the peptidoglycan layer.

A. bisporus is capable of degrading bacteria, *in vitro*, as a sole source of carbon and nitrogen, since extracellular β-*N*-acetylglucosaminidase, protease, lipase and nuclease activities were detected when exposed to non-viable bacteria (Fermor and Wood 1981). *A. bisporus* mycelium can obtain sufficient nutrients from bacteria to grow because of its

slow colonisation. However, other fungi such as weed moulds with high growth rates failed to utilise bacteria as sole carbon and nitrogen source (Fermor and Wood 1981).

Nitrogen can be obtained from the microflora and the compost (inorganic supplements or the ligno-protein complex) (Wood and Fermor 1985). This has been determined by following the extracellular activity of acid, neutral and alkaline proteases, which are associated with mycelial growth of the fungus (Fermor and Wood 1981).

Essential elements required by *A. bisporus* are divided into 2 groups: i) macronutrients, required at *ca.* 10^{-3}M, e.g. calcium, magnesium, phosphorus, potassium and sulphur and ii) micronutrients, required at $\leq 10^{-6}$M, e.g. copper, iron, manganese, molybdenum and zinc (Treschow 1944; Bohus 1959). These nutrients are required for roles in essential structures or metabolic reactions. Calcium is supplied in excess in the form of gypsum ($CaSO_4.2H_2O$) and the other essential elements are present in sufficient quantities.

A. bisporus requires vitamins which function as co-enzymes. Treschow (1944) determined that biotin (B_7) and thiamine (B_1) were essential for growth of *A. bisporus*. The microflora contains a source of vitamins (Stanek 1972). Other nutrients have various roles such as lipids, which could have effects on metabolic pathways involved in linoleate production.

1.1 Parasites and pests of *A. bisporus*

1.1.1 An overview of diseases and pests of A. bisporus

Commercial production of *A. bisporus* appears to be vulnerable to attack from a wide range of diseases and pests. Fungi, bacteria and viruses all cause diseases capable of reducing the mushroom yield.

Verticillium fungicola var. *fungicola* causes the most common and serious fungal disease of mushrooms, dry bubble disease. The reproductive stages alone are susceptible (Gandy 1985). Early infection produces small undifferentiated masses of tissue (up to 2cm) and infection of the later stages of development results in partially differentiated caps or distorted stipes with tilted caps covered in fine, grey-white mycelium (Fletcher *et al.* 1989). Infected mature mushrooms have small pimple-like growths or blue-grey spots (1-2cm dia) and stipes may peel (Fletcher *et al.* 1989). *V. fungicola* produces depolymerases to degrade the host cell wall (Kalberer 1984; Gandy 1985). *V. fungicola* is an invasive

parasite that penetrates the *A. bisporus* cell wall *via* appressorial structures; intracellular *Verticillium* hyphae form, but no haustoria are produced (Dragt *et al.* 1996).

There are many fungal species that develop moulds in mushroom compost and these are usually named according to their colour. Moulds are indicative of ill-prepared composts or conditions that are adverse to mushroom colonisation (reviewed by Fletcher *et al.* 1989). Examples of moulds include *Sepeonium* spp. (yellow moulds) and *Coprinus* spp. (ink caps).

Pseudomonas tolaasii is the causal agent of bacterial or brown blotch, a common and serious disease of *A. bisporus*. Blotches on the cap surface darken with age and in severe outbreaks, mushrooms may be distorted with split caps (Fletcher *et al.* 1989). Infected *A. bisporus* hyphae collapse as the cell contents plasmolyse, however the bacteria remain external and then cluster in the interhyphal spaces (Gandy 1985).

Virus diseases of mushrooms were first described in 1950 (Sinden and Hauser) after a series of crop losses on a farm owned by the La France brothers. Virus symptoms vary but the most consistent symptom is the reduction in mushroom yield (Atkey 1985). Virus particles differ in morphology. Mushroom virus 1 (MV1), spherical with a diameter of 25nm; MV2, spherical with diameter of 29nm; MV3, bacilliform with dimensions 19 x 50nm (Hollings 1962); MV4, spherical with diameter of 35nm; MV5, spherical with diameter of 50nm (Holling *et al.* 1968). Combinations of these virus particles can cause severe crop losses; for example MV1 and MV4 (Fletcher *et al.* 1989).

There are many pests associated with commercial mushroom production; some are considered primary pests, while others cause indirect damage to the crop. The mushroom pests can be divided into flies, mites and nematodes.

Mites often associated with mushroom production are abundant at phase I of composting but should be killed by the peak-heat stage. The mites found post spawning are not usually primary pests and some are predatory towards weed moulds, bacteria or nematodes.

Red pepper mites are of the genus *Pygmephorus* and 3 species have been recorded on mushrooms in UK. These mites are most common during cropping stages of mushroom

production when swarms are observed on surfaces of mushrooms and casing. Red pepper mites selectively feed on weed moulds, in particular *Trichoderma* spp. and their presence of such mites usually indicates poor compost. Red pepper mites have also been associated with the dissemination of *T. harzianum* spores, exacerbating green mould infestations. *Pygmephorus* mites are yellowish-brown and wedge-shaped.

1.1.2 Trichoderma harzianum Colonisation of Mushroom Compost

History

Strains of *T.harzianum* have become aggressive colonisers of *Agaricus* compost. The outbreak of these green moulds in Ireland (1985-1986) had a devastating affect on the yield of mushrooms and problems have also occurred in the rest of the UK. The estimated losses due to green mould infestation were set at £3-4 million for the UK and Irish industries (Mills PR, HRI Wellesbourne, pers. comm.). Previously green moulds were not considered a problem (Fletcher 1986) as those species frequently isolated, *T.viride* and *T. koningii* (Seaby 1996b), did not have the same effect as the subsequent aggressive *T. harzianum* strains.

In 1990 *Trichoderma* green mould outbreaks were reported in Canada and North America (Rinker and Alm 1997b). Green mould problems were reported much earlier in North America (Sinden and Hauser 1953) but these small-scale first incidents were thought to be caused by *T. koningii*. These cases were overcome by sanitation, fungicide application and good pasteurisation and conditioning of compost. The new infestations in North America were caused by particularly aggressive *T. harzianum* strains, and were not controlled by the measures previously used. Economic losses due to *T. harzianum* infestations have been estimated at tens of millions of dollars (Castle *et al.* 1998).

Symptoms

The mycelial growth of *T. harzianum* is sparse and white and after early stages of spawning large patches of compost became green with *T. harzianum* spores. Infestations at this stage were too large to control and the crop was lost. Colonisation of compost by *T. harzianum* caused severely reduced yields and where stacking system was employed, there were cases of cap spotting (Seaby, 1996a). In addition the red pepper mite, *Pygmephorus mesembrinae,* was observed to associate with *T. harzianum* spores. These mites fed on *T.*

harzianum spores and appeared to be a major vector spreading spores (Seaby, 1987, 1996a; Staunton, 1987).

Research into *Trichoderma* green moulds of mushroom compost has focused on the classification of *Trichoderma* isolates, identification of possible sources of contamination and the most efficient measures of control to be practised.

Classification of Trichoderma harzianum strains

Outbreaks of *Trichoderma* infestations of 1985-86 triggered an investigation into the problem (Seaby 1987). Isolations from various colonies found in mushroom compost and strain characteristics were studied (Seaby 1987). Techniques involved measurements of growth rate at different temperature regimes, presence and colour of sporulation and microscopic investigations to determine phialide size, shape, grouping and distribution. Under such criteria seven *Trichoderma* spp. and two *Gliocladium* spp. were recognised to be frequently isolated from mushroom beds. The isolates were further tested for the ability to colonise mushroom compost *in vitro* in plastic screw capped jars (Seaby 1987; Grogan and Gaze 1995). This revealed three strains/micro species of *T. harzianum* that were more successful in colonising compost. The strain termed Th2 was particularly aggressive and managed 100% colonisation of compost (Seaby 1987). Th2 strains were able to produce abundant aerial mycelium in the dark and delayed sporulation, which required greater light intensity than other genotypes. The delayed sporulation tactic was suggested to allow Th2 more time to compete with *Agaricus* and colonise more of the compost (Seaby, 1987). Th2 infestations only occurred in spawned compost. This led to the suggestion of an obligate commensal/parasite relationship between Th2 strains and *A.bisporus* (Seaby 1987).

To overcome discrepancies in strain classification, Seaby (1996a) devised a strict regime for culture conditions. The following culture characteristics were recorded: two growth measurements; form of colony edge; form and degree of sporulation. Observations were made at strict time intervals (Seaby 1996a). Strict culture methods enabled the distinction of groups or genotypes of *T. harzianum* and those classified as Th2 isolates caused the infestations in compost. American isolates constituted a separate genotype termed Th4. Th1 and Th2 displayed the same growth rate and Th3 and Th4 were slower (Seaby, 1996a). Sporulation differed between groups: Th1 – dull green; Th2 – white tufts turning green; Th3 – blue green; Th4 – yellow green. Th3 isolates also had a distinct odour of

coconut, while Th4 had a sour to coconut smell (Seaby 1996a). Phialide size and shape were also specific to genotypes. The taxa of *Trichoderma* described by Seaby (1996a) are in agreement with the molecular typing of Muthumeenakshi *et al.* (1994).

Classification by morphological characteristics alone can reveal some inconsistencies unless strict culturing regimes are adhered to and therefore molecular techniques were employed to provide an accurate and rapid typing procedure for *T. harzianum* strains (Muthumeenakshi *et al.* 1994). Restriction fragment length polymorphism (RFLP) analysis was made of the ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of 81 isolates of *T. harzianum* collected from Irish and UK mushroom industries (Muthumeenakshi *et al.* 1994). Analyses resulted in the recognition of three distinct groups designated Th1, Th2 and Th3. RFLP of rDNA revealed uniform patterns for isolates within a group. Polymorphisms in mtDNA could be distinguished into three clusters which correlated to Th1, Th2 and Th3 of rDNA, however there was also intraspecific variation and therefore isolates were allocated to subgroups. Th1 isolates were divided into 7 subgroups (1a-1g), 1a being by far the largest; Th2 isolates comprised 5 subgroups (2a-2e), 2a forming the majority; and Th3 categorised 5 subgroups (3a-3e). Random amplified polymorphic DNA (RAPD) analysis was used to estimate the intraspecific diversity of randomly chosen *T. harzianum* isolates and confirmed the three major groups revealed by RFLP analyses (Muthumeenakshi *et al.*1994). RAPDs formed three clusters: cluster 1 represented Th1 with similarities of 72-100%; cluster 2 contained Th2 isolates and were identical and cluster 3 had similarities of 70-100% and this represented Th3 isolates (Muthumeenakshi *et al.* 1994). Interspecific comparisons revealed higher degree of similarity between Th1 and Th2 than with group 3 when compared in all combinations (Muthumeenakshi *et al.* 1994). Finally, sequence from the internal transcribed spacer (ITS) 1 region also revealed the same 3 groupings and divergence between these groups was measured as 4.7% divergence of group 1 from 2 and groups 1 and 2 showed 20.0% and 22.9% divergence from group 3 respectively (Muthumeenakshi *et al.* 1994).

Similar molecular techniques were used to determine the genotypes of North American aggressive *T. harzianum* strains to trace the origin of these strains. Techniques revealed that Th1 is the most recent ancestor for Th2 and Th4 (Ospina-Giraldo *et al.* 1997a, 1998) however, both aggressive genotypes are genetically distinct to Th1 (Muthumeenakshi *et al.* 1998). There are various hypotheses as to the source of aggressive *T. harzianum* strains: (i)

a mutant particularly suited to colonising mushroom compost as a result of multiple mutational events seen as the mitochondrial polymorphisms; (ii) the artificial conditions of mushroom production led to strong selection for a specific type of *T. harzianum* from existing populations (Castle *et al.* 1998); (iii) the evolution of an aggressive strain from a closely related, non-aggressive due to recent speciation within an isolated, genetically homogeneous sub-population (Muthumeenakshi *et al.* 1998).

Molecular comparison between the aggressive genotypes of *T. harzianum* and strains of *T. harzianum* used in biocontrol in America, confirmed that biocontrol species were not the source of aggressive strains (Romaine *et al.* 1997).

Epidemiology

The possible infection routes and the possible conditions necessary for *Trichoderma* infestations have been investigated as a means to eradicate or at least, reduce the extent of the problem (Seaby 1987, 1996b; Staunton 1987; Grogan and Gaze 1995; Fletcher 1997).

Possible infection routes included the following areas: faulty peak heating of compost, dust particles, contaminated clothing, animal vectors, airborne infection, infected spawn, surface spawning, contamination of compost by handling, mixing infected spawn run compost into casing and machinery, equipment and trailers (Seaby 1987, 1996b). Faulty peak heating of compost was eliminated as no *Trichoderma* propagules survived the heat cycles of 58-60°C followed by 46°C (Seaby 1987). Lagoon water used on mushroom farms harboured *T. pseudokoningii, T. viride, T. longibrachiatum* and Th1 and Th3 *T. harzianum* spores only (Seaby 1987, 1996b).

Dust particles were a source of infection under certain weather conditions (i.e. dry and windy). Failure to dispose of contaminated waste, especially at peak heating tunnel exits, also resulted in disease spread (Seaby 1987). Contaminated clothing led to passage of the disease and samples yielded almost pure cultures of Th2 (Seaby 1987, 1996b). Airborne infestations were possible in the right conditions, for example where infected compost was removed but left in the vicinity of air inlets and doorways (Seaby 1987).

Animals such as mites, sciarid flies and mice were capable of dispersing *Trichoderma* spores (Seaby 1987, 1996b). Mites were suggested to have a host-vector relationship with

Th2 strains because the former were attracted to Th2 mycelium and subsequently fed on Th2 spores, which also adhered to their bodies. In addition, feeding of *P. mesembrinae* on *Trichoderma* had a stimulatory effect on the mycelial growth (Terras and Hales 1995). Terras and Hales (1995) reported the presence of sporothecae on the red pepper mite, used for carrying spores; this structure is a shallow invagination next to leg IV and it is capable of carrying up to 9 conidia (Terras and Hales 1995). Th2 spores also adhered to sciarid wings and mice carried Th2 spores and mites (Seaby 1987, 1996b).

Both contaminated spawn and surface spawning hastened the infection as in each case Th2 spores were allowed direct access to an initial nutrient base (Seaby 1987). *Trichoderma* contaminated spawn spread very quickly through the compost bag but also contaminated the spawn hopper and clean spawn as it entered the hopper (Seaby 1987). Damage to the spawn bag by handling, mice and oviposited fly eggs were all possible routes of infection. Cross contamination by growers who handled infected spawn run compost and then moved to fresh compost areas revealed a direct route of infection as did mixing infected spawn run compost into casing. Finally, contaminated machinery (i.e. conveyors and vehicles used for emptying and refilling houses), equipment (i.e. thermometers moved between bags) and trailers (i.e. spread contaminated compost back to compost producer) were all sources of infection (Seaby 1987).

Identification of *Trichoderma* species detected in compost production yards and mushroom growing units in Ireland, revealed classification of morphological and growth characteristics as previously found by Seaby (1987) (Morris *et al.* 1995a, 1995b). Th1 was most frequently found in the compost production yards, however Th2 was the cause of green mould problems. Compared with other species of *Trichoderma* (*T. viride, T. longibrachiatum* and *T. koningii*) found on the compost production yards, *T. harzianum* was the most common (Morris *et al.* 1995a). The studies directed towards *Trichoderma* species within the mushroom units also indicated that *T. harzianum* was the most frequently observed and that Th2 comprised the vast majority of the isolates, at 76% (Morris *et al.* 1995b).

Rinker *et al.* (1997) reported similar studies of epidemiology for Th4 isolates to trace possible infection sources and methods of spreading the infections confirming the work of Seaby (1996b). Th4 isolations were much higher inside, rather than outside, buildings on

mushroom farms and the highest levels of contamination were found in areas common to personnel e.g. washrooms and lunchrooms (Rinker *et al.* 1997). Th4 spores could survive the post-crop cook-out (high temperatures to eliminate contaminants) and therefore suggested that spent compost should be steamed at 65°C for over 24 hours (Rinker *et al.* 1997). Finally, Th4 incident levels were observed to differ according to the stage of mushroom production; significant levels of Th4 coincided with the spawning and casing operations (Rinker *et al.* 1997).

Factors influencing Trichoderma infestations

Although some patterns had emerged as to some of the conditions necessary for *Trichoderma* green mold infestations, these were by no means consistent and so a number of studies have attempted to reveal factors that influence the problem (Seaby 1987, 1996b; Fletcher 1997; Grogan and Gaze 1995).

The influence of moisture and nitrogen content of the compost and its pH revealed no apparent effect on the *Trichoderma* infection (Seaby 1987; Staunton 1987). However there are exceptions to this; particularly heavy *in vitro T. harzianum* infestations were linked to compost types with moisture content below 65% and occasionally, high moisture and nitrogen contents have resulted in severe infestations. Poorly prepared compost was susceptible to increased infection, probably due to higher concentrations of carbohydrate and lower levels of bacteria (Seaby 1987). The addition of essential metals such as copper, zinc and iron failed to enhance *A. bisporus* and there was no reduction in the level of *T. harzianum*, in fact an increase was detected (Seaby 1996b).

Grogan and Gaze (1995) studied crop yields from a range of 8 experimental and 4 batches of commercial compost all previously exposed to *T. harzianum* spore suspensions. Commercial composts produced the best yields. Three of the experimental composts provided the lowest yields, however there was no single factor attributable; low aeration combined with higher/lower than standard temperature levels and pH appeared to be responsible (Grogan and Gaze 1995).

The inoculum level of *T. harzianum* was also investigated and experiments revealed that there was a significant increase in *Trichoderma* propagules recovered from compost as the initial inoculum concentration was increased (Grogan and Gaze 1995). Inconsistencies

with this trend did occur; one of the experimental composts suffered the largest reduction in crop yield yet recovered the smallest *Trichoderma* populations (Grogan and Gaze 1995) and therefore disease incidence is not depend on the inoculum level alone.

The manner in which *T. harzianum* entered the compost was important. When inoculated as a spore suspension to compost in the absence of spawn grain or autoclaved wheat grain, no infestations occurred. The presence of spawn grain and autoclaved wheat grain caused an increase in *Trichoderma* populations (Fletcher 1997). By comparing spawn, autoclaved cereal grain and vermiculite as *Trichoderma* inoculum carriers, it was revealed that the cereal grain appeared to act as a nutritional base (Fletcher 1997). The distance between the inocula influenced the outcome since placed adjacent to the spawn grain, *T. harzianum* was capable of significantly reducing colonisation by *A. bisporus* regardless of the inoculum carrier of *T. harzianum*. At a distance of 50mm from the spawn grain, the cereal-based *T. harzianum* inoculum, also significantly reduced *A.bisporus*, although the effect was reduced and inoculum on the vermiculite carrier had no effect (Fletcher 1997). Thus *T. harzianum* development in compost appeared to depend on the presence of an initial nutrient source. Contrary to this, Seaby (1987) reported on the importance of mushroom mycelium for the green mould infections to occur since it was impossible to infest fresh compost.

Germination and growth of Th2 was reported to be inhibited by the presence of bacterial colonies *in vitro* (Seaby 1996b) and this may suggest that the ability of *A. bisporus* to lyse bacteria could remove inhibitory effects of micro-organisms towards *T. harzianum*.

Higher temperatures than usually employed in commercial production may be associated with increased damaging effects of *T. harzianum* on *A. bisporus* (Seaby 1987, 1996b; Fletcher 1997). Even Th1 strains not usually connected with green mould infestations showed detrimental effects towards *A. bisporus* at 30°C (Seaby 1987, 1996b). Th2 strains were detrimental across the temperature range. The aggressiveness of Th4 also differed according to the temperature; a positive correlation was observed between an increase in temperature and an increase in Th4 spot size (Rinker and Alm 1997b; Romaine *et al.* 1997). However, mushroom yields were greatly reduced in composts infested with Th4 regardless of temperature (Rinker and Alm 1997b).

Fletcher (1997) recorded growth rates of *A. bisporus* (from spawn) and Th2 strains at 10, 15, 20, 25 and 30°C. *T. harzianum* grew at all temperatures and *A. bisporus* also showed dense mycelial growth across the temperature range except at 15°C (Fletcher 1997). This delay in *A. bisporus* growth rate was suggested to provide *T. harzianum* with the opportunity to colonise the spawn grain before *A. bisporus* had produced a "protective" mycelial barrier (Fletcher 1997). Furthermore, early inoculation (prior to spawning) with Th2 resulted in heavy infestations and no colonisation occurred when Th2 was inoculated after the establishment of *A. bisporus* mycelium.

Growers in North America noticed that brown strains of *A. bisporus* supported less *T. harzianum* green mould. Experiments confirmed a reduction of green mould spot size of between 50 and 75% in brown strains compared to white (Rinker and Alm 1997b). Brown strains produced a mushroom crop albeit reduced, however white strains suffered complete loss of yield when infested with Th4 (Rinker and Alm 1997b).

Prevention and control of T. harzianum outbreaks

Reduction and prevention of *Trichoderma* green mould infestations has been achieved by increased levels of hygiene (Seaby 1987, 1996b; Staunton 1987; Fletcher 1989; 1997; Rinker and Alm 1997b; Rinker *et al.* 1997). This includes measures to avoid contamination by dust, such as enclosed bagging areas and prevention of contact between workers in bagging areas and pickers, whose overalls should be regularly sterilised (Seaby 1987).

Biological control has been suggested, for example two biological bacterial "fungicides" GB29 and GB03E (Gustafson, Dallas, TX) which achieved a reduction of more than 60% and 80% respectively in Th4 incidents (Romaine *et al.* 1997; Ospina-Giraldo *et al.* 1997c). These biological controls are too expensive to be a viable option.

Benomyl failed to control Th2 although a reduction of infection was observed (Seaby 1987). The comparison of three benzimidazole fungicides: Benlate, Bavistin DF and H250 (liquid formulation of Thiabendazole) revealed significant control of *T. harzianum* with no deleterious effects towards *A. bisporus* yields by Bavistin DF (Grogan and Fletcher 1993; Grogan and Young 1995). Spawn applications demonstrated the best control. To increase the sanitation of wood surfaces and floors, the fungicide Terraclor® was effective (Romaine *et al.* 1997).

Potential threat of T. harzianum Th4 to other species

Th4 caused a *Pleurotus ostreatus* (oyster mushroom) crop loss of over 77% in America (Royse *et al.* 1998). Production of oyster mushroom has increased extensively in the last decade and now 797,000 tonnes are produced worldwide (Royse *et al.* 1998). Oyster mushrooms are grown in chopped wheat or oat straw [pH 7.5] (Stoltzer and Grabbe 1991). The green mould infestation was classified as Th4 by molecular techniques (Royse *et al.* 1998). Therefore, Th4 strains are adaptable and capable of colonising different substrates under similar conditions.

Lentinula edodes exhibits resistance towards Trichoderma species.

Trichoderma harzianum attacks the bedlogs of *Lentinula edodes* (shiitake mushroom) along with *T. polysporum*. Both species of *Trichoderma* produce a range of antifungals and depolymerases, but some strains of *L. edodes* are resistant to these chemicals (Tokimoto *et al.* 1995). The resistant strains of *L. edodes* have been shown to produce straight-chain alcohols with double and triple bonds that possess antifungal behaviour. The resistance to attack by *T. harzianum* was hereditary and dominant (Tokimoto et al, 1995). When cultivated on wood-powder medium the resistance was comparable to that observed on the bedlogs, therefore the *in vitro* method could be used to select and breed for resistance.

An assay has been developed to screen strains of *Lentinus edodes* for resistance to *Trichoderma harzianum* (Ohmasa *et al.* 1995). Small blocks of *L. edodes* mycelium were sprayed with condia of *T. harzianum* and the disease incidence was evaluated after incubation. A medium that contained casamino acids increased the antagonistic effect of *T. harzianum* on *L. edodes*, as did a high *T. harzianum* spore concentration, higher temperatures (30°C) and high relative humidity (Ohmasa *et al.* 1995). Another assay for the selection of shiitake strains that are competitive to *Trichoderma* spp. has been described by Tokimoto *et al.* (1998). Dual cultures of *L. edodes* and *Trichoderma* spp. were cultured on sawdust medium with 1/3 volume of rice bran. The selection assay provided an estimation of competitive abilities of dikaryotic strains of *L. edodes*, which were also capable of repelling *Trichoderma* in outdoor log cultivation.

The relationship, if any, between *T. harzianum* and *A. bisporus* remains undetermined but has been described as obligate commensal/parasite (Seaby 1987, 1996b) and competitive

(Fletcher 1997). Characteristics of *T. harzianum* that could be detrimental to the mushroom industry include the production of many antibiotics and degradative enzymes and an ability to out compete several other fungi perhaps including *A. bisporus*.

1.2 Antagonism and *T. harzianum*

Antagonistic interactions can be extremely complicated involving many resources and these include nutrients, water and space. Competition and mycoparasitism will be described in this section. In both situations one organism benefits at the expense of another, however they differ in the resources involved. The significance of the discussion on antagonism concerns the relationship between aggressive genotypes of *T. harzianum* and *A. bisporus*, which from previous investigations would suggest the involvement of antagonism. Techniques to study the various forms of antagonism have been designed for laboratory investigations and these can only be considered as simplistic models. In nature, the situation is far more complex and fluctuations in environmental factors influence the outcome of an interaction, which may change continually with time and with the constantly varying environment.

The mode of life of fungi is very important. Many researchers have described the various modes of fungal life including Cooke and Rayner (1984), Deacon (1984) and Deverall (1981); which have been used to compose this section. Fungi obtain nutrients from living or non-living substrates. Fungi that feed from dead organic material are termed 'saprotrophs' and are often referred to as the 'decomposer' fungi. Parasitic fungi obtain nutrients from a living 'host'. Fungal parasites can be further divided into those that kill and destroy their hosts, termed 'necrotrophs' and those that require living hosts, 'biotrophs'. The descriptions of these terms tend to suggest these modes of life are exclusive, however, Cooke and Rayner (1984) state that biotrophy, necrotrophy and saprotrophy are not necessarily mutually exclusive. This adds further complexity to investigations of possible interactions, as the antagonist may exist in more than one mode of life and shift between different antagonistic interactions.

1.2.1 Competition Theory

The concept of competition has triggered various definitions, depending on the types of organisms studied and the environment in which an interaction is occurring. Keddy (1989) suggests that while there is no coherent body of competition theory, the concept of

competition can help scientists to organise and understand nature, as well as, predict and foresee the world accurately. A definition of competition between organisms that fits the fungal community is that described by Keddy (1989): "the negative effects which one organism has upon another by consuming, or controlling access to, a resource that is limited in availability". Therefore, when studying possible competitive interactions due consideration must be given to the resources involved, the mechanisms of competition and the individuals or groups competing. Competitive strategies and mechanisms have been comprehensively reviewed by Widden (1997); other aspects of competition, including resources, have been covered by Keddy (1989), Cooke and Rayner (1984) and Tilman (1982) and it is from these texts that the following section has been composed.

Resources

The resource(s) responsible for a competitive relationship must be understood in order to study such an interaction. Tilman (1982) defines a resource as "any substance or factor which is consumed by an organism and which can lead to increased growth rates as its availability in the environment is increased". It is from this definition that some researchers argue that competition between organisms must be proven by demonstration of a resource limitation. Resources involved in competition interactions are nutrients, water, space and hosts (Widden 1997). The basic nutrient requirements of all living organisms are for carbon, hydrogen, nitrogen, oxygen, magnesium, potassium, manganese, phosphorus and sulphur, however the kinds of resources these elements are found in will differ. Price (1984) described four situations that could effect resources: 1) trophic positions, 2) temporal and spatial distribution, 3) mode of consumption and 4) resource ratios.

Organisms may be separated according to their trophic group. Autotrophs are capable of assimilating carbon dioxide into complex carbohydrates; some may use sunlight as an energy source for this process. Heterotrophs require fixed carbon and so acquire carbohydrates from other organisms. Thus the resources used will differ according to the trophic level of the organism.

Resources also vary with time and Price (1984) devised five classes of resources. "Increasing resources", increase over the active season of an organism and then suddenly decline. "Decreasing resources", are produced suddenly at the start of a season and gradually decline. "Pulsing or ephemeral resources" increase rapidly, followed by a rapid

decrease. The "steadily renewed resources" are continuously renewed over long periods of time and "constant resources" are physical in nature and not affected by seasonal change. Southwood's (1977) theory is more complex and involves four temporal and three spatial categories. Temporal categories are resource at a constant high, seasonal availability, unpredictable availability and ephemeral. Spatial categories for resource availability are continuous, patchy (analogous to seasonal) and isolated (analogous to ephemeral). Keddy (1989) suggests that there may also be a constant but chronically low resource supply rate, which was not mentioned by Price and Southwood.

The mode of consumption of a resource can be divided into two categories (Yodzis 1986). Organisms that harvest a fraction of a resource over a large area are said to be in consumptive competition. Space competition occurs when organisms harvest all of a resource from a fraction of the total resource area. The mode of consumption determines the organisation of the community; consumptive results in organisation by resource partitioning and space competition results in hierarchies of dominance.

Organisms require more than one resource to survive and reproduce and therefore, if a particular resource is limiting another resource may replace it. Organisms were tested for responses to different ratios of resources (Tilman 1982). Tilman (1982) suggested that there are two types of resources, those that are replaceable or 'substitutable' and those that are the sole source or 'essential' resources. Organisms that used substitutable resources still maintained a constant growth rate. Substitutable resources were divided into three groups: 1) perfectly substitutable, can survive on one or another resource with no change in growth rate; 2) complementary resources, which have a synergistic effect and the mixtures enhance growth rate; 3) antagonistic resources, interfere with one another and the mixture reduces growth rates (Keddy 1989).

The immense varieties in which resources exist further indicate the complexity of interactions between organisms in nature. This reiterates the importance of understanding the resources present in the environment and the relevant importance of the individual resources to the organisms involved in possible interactions.

Strategies of Competition

Various strategies for competition have been proposed including r- and K-selection (Pianka 1970) and ruderal, competitive and stress-tolerant strategies (Grime 1977). The strategies describe different environments and the traits that are responsible for successful organisms within the environment. Pianka (1970) suggests that r-selected organisms are opportunistic and are found in environments that are regularly disturbed. Opportunistic organisms are characterised by their fast growth rates and short life-spans; choosing to channel energy into reproduction as an escape mechanism to new resources. Populations in equilibrium are classified as K-selected organisms (Pianka 1970) and are found in stable and undisturbed environments. K-selected organisms tend to be large and have slow growth rates. These organisms direct energy into biomass production and competitive mechanisms. In the situation of *A. bisporus* and *T. harzianum* from the outset, the former is K-selected and the latter could be considered as r-selected.

Grime (1977) devised three strategies for plants and these were based on four extremes of habitat: low stress/low disturbance; low stress/high disturbance; high stress/low disturbance; and high stress/high disturbance. The three strategies to cope with these environmental conditions were: competitive (C-selected), adapted to low stress/low disturbance; ruderal (R-selected), adapted to low stress/high disturbance; and stress-tolerant (S-selected), adapted to high stress/low disturbance. Grime suggested that plants were not capable of adapting to high stress/high disturbance environments. As these strategies are generalised and often devised on the basis of a particular organism, adaptation of the strategy to a completely different organism can reveal flaws that must be considered carefully. Keddy (1989) argues that the ruderal, competitive and stress-tolerant strategies are contradictory. This strategy suggests that competition is high in a resource-rich environment, however by the previous definition (Keddy 1989) competition is the result of a limiting resource. The disagreement referred back to the organism on which Grime's strategy was based. Grime's strategy was based on competition between plants and no distinction was made between competition for light and space (above ground) and water and nutrients (below ground). Competition between plants for energy (light) will be high when roots are in nutrient-rich environments. This demonstrates the significance of understanding the full implications of a proposed strategy when dealing with competitive interactions between organisms.

Mechanisms of Competition

There are two recognised types of competition (reviewed by Keddy 1989; Widden 1997). "Interference" competition occurs when two fast growing organisms come into direct contact either chemically (toxins) or physically (forcing the other organism in another direction). When competing, a dominant fungus may interfere with another fungus using a number of different techniques including the production of antimicrobial compounds, secretion of depolymerases and direct physical attack such as hyphal interference and penetration of the competing organism (Widden 1997).

When an organism has an indirect competitive effect on another organism as a result of resource depletion, "exploitation" competition has occurred. Exploitation competition can be demonstrated by two or more organisms requiring the same resource; the organism that uses the substrate most efficiently will outcompete the others (Tilman 1982). The change in characteristics of the substrate is demonstrated by a succession of organisms. Decomposition of a substrate leads to changes, which may be better suited to a different organism and the replacement organism is termed the successor. Garrett (1963) described the succession of fungi on a substrate; successors required high competitive saprophytic ability (CSA). "Sugar fungi" were suggested to colonise first and remained dominant while readily utilised carbon sources were available, due to their ability to exploit these resources more efficiently than other fungi. Sugar fungi were succeeded by fungi with a versatile enzyme system, capable of degrading complex carbohydrates such as hemicelluloses and cellulose. Finally the Basidiomycetes, capable of degrading recalcitrant materials such as lignin, were the last to colonise plant matter resources.

Chemical interference in competitive interactions has been suggested in many systems, however there is little evidence that such compounds are produced in the natural environment and even less to suggest that they are directly involved in antagonism (Widden 1997). Three types of physical interference have been recorded both in culture and nature, these are: 1) hyphal barrages or dense hyphal growth at contact point, 2) overgrowth of one colony by another or 3) hyphal coiling (Widden 1997).

The study of competitive interactions between fungi requires an understanding of the nature of the competition, the type of resource competed for and the long-term effects of various organisms on the densities of their resources (Widden 1997).

Trichoderma species as competitors

A competitive interaction between fungal communities can be difficult to expose in nature, however the literature provides several interesting examples. *Trichoderma* species are ubiquitous in most soil types and are antagonistic towards many fungal species (Chet 1987). The competitive strategy of *Trichoderma* species can appear disconcerting; the nature of their rapid growth rate and production of vast numbers of propagules could class *Trichoderma* as ruderals. However, these micromycetes exhibit a versatile complement of lytic enzymes suggesting a more combative strategy (Widden 1997). Thus indicating that the *Trichoderma* have the capability to switch strategies according to environmental conditions and community structure and explain their ubiquity within soils in nature. Widden and Scattolin (1988) suggested that *Trichoderma* species should properly be considered as C-selected by virtue of the depolymerases they secrete. Combative fungi replace ruderals on a substrate and the advantage of overlapping the two strategies possibly allows *Trichoderma* spp. to withstand invasions from other combative fungi. *Trichoderma* species have been investigated for intraspecific and interspecific competition in nature.

The competitive interactions between species of *Trichoderma* when colonising spruce litter revealed large variations in competitive strategies (Widden and Scattolin 1988). *T. hamatum, T. koningii, T. viride, T. polysporum* and isolate LP58 (a species similar to *Gliocladium virens*) were studied and *Trichoderma* species were observed to change in abundance according to the season (Widden and Abitbol 1980). Therefore, Widden and Scattolin (1988) questioned the ability of species to compete intraspecifically and the effect of temperature on this process. *T. polysporum* and *T. viride* exhibited a stress-tolerant strategy, whereas *T. hamatum* and *T. koningii* displayed a strategy more combative in nature. A ruderal strategy was employed by isolate LP58, which had the fastest growth rate, most rapid sporulation and exhibited poor competitive colonisation (Widden and Scattolin 1988). At lower temperatures (5 and 10°C) *T. polysporum* and *T. viride* were the most competitive even though they had slow growth and sporulation rates (S-selected). At higher temperatures, the C-selected *T. hamatum* and *T. koningii* were the most competitive and were not easily replaced, tending to retain possession and replace the other species. This reflected the situation in nature as in warmer seasons *T. hamatum, T. koningii* and LP58 were in abundance in spruce litter and these were replaced by *T. polysporum* and *T. viride* in the cooler months (Widden and Abitbol 1980).

Trichoderma species have also been observed in competitive interactions with other genera. Wardle and coworkers (1993) observed interactions between *T. harzianum* and *Mucor hiemalis* in agricultural soil and between *T. polysporum* and *M. hiemalis* in forest litter. In both environments *T. harzianum* and *M. hiemalis* were capable of coexistence since neither species was outcompeted to the extent of extinction. In agricultural soil *M. hiemalis* outcompeted *T. harzianum*, however *T. polysporum* competitively inhibited *M. hiemalis* in forest litter. The types of resources available and the various competitive strategies of the fungi involved appear to be responsible. Agricultural soil lacked complex carbohydrates and therefore *M. hiemalis* as a true ruderal was well adapted to exploit simple sugar sources more efficiently than the combative *T.harzianum*. Conversely forest litter with abundant cellulose enabled *T. polysporum*, with a complex cellulolytic system, to outcompete *M. hiemalis*.

In each of these examples species of *Trichoderma* inhabited different niches and experienced different environmental conditions. Species of *Trichoderma* adopt very different competitive strategies and in some cases, such as *T. polysporum*, they may change strategies according to the environmental pressures, reinforcing the fact that environmental influences lead to successions or seasonal changes.

1.2.2 Mycoparasitism Theory

Mycoparasitism has been defined as "the direct attack on a fungal thallus, followed by nutrient utilization by the parasite" (Chet *et al.* 1997). The only resource concerned in this type of interaction is nutrients. There are two types of mycoparasitism; necrotrophs kill the host to obtain nutrients while biotrophs acquire nutrients from living hosts. The destructive necrotroph has a wider host range compared to biotrophs since the necrotroph does not require specialised interfaces to obtain nutrients. Necrotrophs must maintain close contact with their hosts to avoid competition from saprotrophs. Biotrophic mycoparasites employ subtle techniques to ensure that the host remains viable and are therefore ecologically obligate parasites (Deverall 1981; Chet *et al.* 1997; Jeffries 1997). Nutrients are obtained by distinct infection structures used to invade the host and subsequently form interfaces through which, nutrients are absorbed with no apparent impairment to the host. Biotrophic mycoparasites may be grouped according to the interfaces produced (Table 1.1).

To obtain nutrient resources mycoparasites produce antibiotics, toxins and lytic enzymes. In addition some parasites coil around host hyphae, which are frequently penetrated. One or all of these mechanisms may be employed by a mycoparasite to parasitize successfully its host (Jeffries 1997). These same mechanisms may also occur during a competitive interaction and therefore it can be difficult to distinguish a competitive from a parasitic interaction. Interactions that demonstrate one fungus obtaining nutrients directly or indirectly from another fungus can be classified as mycoparasitism (Jeffries 1997). Other complications arising from terminology include the fine boundaries between necrotrophy and saprotrophy. A necrotrophic mycoparasite may detect a potential host and proceed to parasitize it. During the process of necrotrophic parasitism the parasite, by definition, kills its host. Once the parasite has killed its host, the necrotroph could be described as a saprotroph (Jeffries 1997).

There are three recognised steps to the process of mycoparasitism. Initially the parasite undergoes directed growth, probably along a chemical gradient, towards a potential host and this is followed by a recognition reaction. The result of recognition is irreversible attachment to the host surface and subsequent colonisation (Chet 1987; Baker 1987; Manocha and Sahai 1993). The recognition process in fungal-fungal interactions may involve lectin-carbohydrate interactions and these have been comprehensively reviewed (Manocha and Sahai 1993). Recognition results when a complementary interaction occurs between host and parasite cell surfaces. The mycoparasite then inflicts damaging changes on its host, the extent of which is determined by the mode of life of the parasite. Some damages may occur due to secondary metabolites produced and directed towards the host, which are frequently induced during the recognition process. The metabolites may be produced in advance of contact between host and parasite; such techniques are employed to predispose the host (Baker 1987). In other cases, the host cell changes may only occur after contact and formation of infection structures (Baker 1987). On contact with a host, mycoparasites may establish by parallel growth along host hyphae. However, some mycoparasites (e.g. *T. harzianum*) not only produce appressoria-like structures to penetrate, but also coil around the host hyphae. This coiling effect is not a thigmotrophic response (Dennis and Webster 1971c). Nylon threads of diameters equal to host fungi

Table 1.1 Mycoparasites defined by their host-parasite interface (adapted from Jeffries 1997).

Nature of Mycoparasite	Comments
Necrotrophs	
Contact	Grow in close contact with host hyphae Penetration not observed
Invasive	Penetrate host hyphae Invasive growth Result in necrosis and hyphal lysis
Biotrophs	
Haustorial	Penetrate host hyphae by short hyphal branches Haustorium forms and host plasmalemma invaginates around this structure
Intracellular	Penetrate host hyphae and protoplast Parasite thallus enters invaded host cytoplasm
Fusion	Walls of host and parasite form a closely associated contact zone No penetration of host Intracellular channels form connecting protoplasts of host and parasite

failed to induce mycoparasites to coil unless threads were pre-treated with a lectin similar to that present on the host cell walls (Elad *et al.* 1983b; Chet 1987).

T. harzianum isolates have been identified as both competitive saprophytes in soils and as aggressive mycoparasites of several economically important phytopathogens. There is significant evidence for disease reduction as a result of antagonism inflicted by *T. harzianum* (Elad et al 1980, 1982, 1983a; Geremia et al 1993; Inbar et al 1995; Benhamou and Chet 1993, 1996; Vasseur et al 1995; Flores et al 1997).

T. harzianum as a mycoparasite of phytopathogens

T. harzianum can colonise a number of environments successfully as a saprotrophic competitor, however some strains are aggressive mycoparasites of *Rhizoctonia solani* and *Sclerotium rolfsii*. Other hosts of this parasite include *Botrytis cinerea* (Lorito et al 1996), *Pythium ultimum* (Sivan and Chet 1989; Benhamou and Chet 1997) and *Fusarium oxysporum* (Cherif and Benhamou 1990). As previously described, mycoparasitism can proceed *via* several different mechanisms some of which, the use of antibiotic compounds for example, make it difficult to differentiate between antibiosis and mycoparasitism. It appears that both mechanisms may be engaged by a parasite. Therefore, parasitism of *R. solani* and *S. rolfsii* will be discussed here as in both systems a typical necrotrophic mycoparasitic role for *T. harzianum* has been demonstrated.

Mycoparasitic interactions between Trichoderma harzianum and Rhizoctonia solani

Rhizoctonia solani is an important phytopathogen responsible for brown-girdling root rot, damping-off and seedling blight in many crops. Antagonistic properties of *T. harzianum* have shown significant reductions in disease incidence and this mycoparasitic interaction has been researched in some depth as a putative biocontrol.

Trichoderma harzianum recognises hyphae of susceptible *R. solani* by a lectin-carbohydrate interaction (Elad et al 1983b; Barak et al 1986). A lectin, specific for L-fucose, is produced by *R. solani* on the cell surface, as shown by the attachment of type O erythrocytes to *R. solani* hyphae (Elad *et al.* 1983b). Evidence for the complementary L-fucose on *T. harzianum* was provided by failure of the mycoparasite to attach and coil around hyphae of *R. solani* pre-treated with L-fucose. The presence of L-fucose on *T. harzianum* cell surface was further supported by the attachment of fluorescein

isothiocyanate labelled *Lotus tetragonolobus* agglutinin and *Tetragonolobus purpureas* agglutinin; both agglutinins are specific for L-fucose (Barak et al 1986). Ultrastructural studies and gold-complexed labelling revealed another potential lectin of *T. harzianum* origin (Benhamou and Chet 1993). An extracellular matrix, rich in galactose, covers *R. solani* hyphae. The matrix represents the first layer of contact with the mycoparasite and it adheres to *T. harzianum* during the interaction. This suggests the presence of a galactose-specific receptor on the cell surface of the parasite.

Ultrastructural studies of the interaction between *T. harzianum* and *R. solani* have confirmed the nature of the interaction to be mycoparasitic (Elad et al 1983a; Benhamou and Chet 1993). Disorganisation of host cytoplasm and inhibition of growth occurred post contact with parasite and therefore was suggested to be a consequence of the hyphal interference. *T. harzianum* exhibited chemotropic growth towards *R. solani* and proceeded to coil around the host hyphae, which it frequently penetrated. Initially, after contact, *R. solani* maintained cell wall integrity although slight retraction of the plasmalemma was detected. As the interaction progressed the wrinkled appearance of the parasitized hyphae suggested a reduction in turgor. Finally, mycoparasitism resulted in the complete loss of turgor of the host resulting in cell wall breakdown, cytoplasm aggregation and hyphal disintegration.

Penetration of the host hyphae is thought to result from the synergistic action of depolymerising enzymes and mechanical pressure (Elad *et al.* 1983a). This was supported by the accumulation of vesicles and mitochondria in the penetrating hyphae, which are indicative of high metabolic activity. The activity of hydrolytic enzymes directed at *R. solani* cell walls was further corroborated using gold-complexed wheat germ agglutinin (WGA) (Benhamou and Chet 1993). Gold-complexed WGA binds specifically to *N*-acetylglucosamine and when applied to parasitized *R. solani,* revealed irregular binding which verified cell wall chitin degradation. Several cell wall-degrading enzymes, including chitinase (Elad *et al.* 1982; dal Soglio *et al.* 1998), β-1,3-glucanase (Elad *et al.* 1982; dal Soglio *et al.* 1998), proteinase (Geremia *et al.* 1993) and lipase (Benhamou and Chet 1993), have been implicated in the mycoparasitism of *R. solani*. Labelling of *N*-acetylglucosamine in the host cell wall as the interaction progressed revealed gradual degradation of the cell wall (Benhamou and Chet 1993). The gradual release of chitin

monomers which may act as inducers of chitinase production, would ensure continued chitinase activity in a controlled manner.

A serine proteinase (Prb1), secreted in the presence of *R. solani* cell walls, has received significant attention and appears to have a major role in the mycoparasitism of *R. solani* by *T. harzianum* (Geremia *et al.* 1993; Flores *et al.* 1997). Both *R. solani* cell walls and chitin induce Prb1 and expression is controlled at the mRNA level (Geremia *et al.* 1993). The proteinase is synthesised as a pre-proenzyme with a signal peptide for extracellular secretion and its possible roles include degradation of host cell walls, membranes and proteins released after host lysis (Geremia *et al.* 1993). The importance of Prb1 in controlling disease incidence caused by *R. solani* was further confirmed by increasing the gene copy number (Flores *et al.* 1997); this resulted in significantly increased expression of the *prb1* gene and Prb1 activity leading to significantly improved biocontrol compared with that effected by wild type *T. harzianum*.

The gene (*inda1*) of a mycoparasitism-related protein was induced by the presence of *R. solani* was cloned and was shown to be regulated at the mRNA level (Vasseur *et al.* 1995). *Inda1* encoded a putative amino acid permease and was suggested to be embedded in the mycoparasite membrane (Vasseur *et al.* 1995). Possible roles for this protein include the up-take of degradation products of Prb1.

In addition to induction of mycoparasitism-related proteins, on the commencement of mycoparasitism certain protein synthesis is down-regulated. Glyceraldehyde-3-phosphate dehydrogenase (gpd) considered a constitutively expressed gene is repressed during conidiation and mycoparasitism (Puyesky *et al.* 1997). A significant decrease in the abundance of *gpd* mRNA coincided with sporulation and growth in the presence of *R.solani* cell walls. Puyesky *et al.* (1997) suggested that this tightly controlled regulation may partially control the switch to sporulation or mycoparasitism.

Host cell alterations do not commence until after the parasite contacts the host, suggesting that toxins or antibiotics were not deployed to predispose the host. Therefore cell alterations were a direct result of hyphal contact or penetration causing an internal osmotic imbalance (Benhamou and Chet 1993). However in the field and under different *in vitro*

conditions, one cannot rule out the possibility of toxic metabolites used in an antibiosis mechanism.

Mycoparasitic interactions between Trichoderma harzianum and Sclerotium rolfsii

Sclerotium rolfsii is responsible for a range of seedling diseases, including damping-off, of many crops. It is one of the most widely distributed and destructive of pathogens (Papavizas and Lewis 1989). The presence of *T. harzianum* resulted in a significant reduction in the symptoms exacted by *S. rolfsii* (Wells *et al.* 1972). *T. harzianum* could parasitize both mycelium and sclerotia of *S. rolfsii*, as opposed to traditional fungicides, which were incapable of killing sclerotia.

Recognition of *S. rolfsii* by *T. harzianum* is thought to involve an agglutinin on the host cell surface that is specific for D-glucose and D-mannose (Barak *et al.* 1985). Inbar and Chet (1992) confirmed the role for a D-glucose/D-mannose-specific agglutinin since *T. harzianum* attached and coiled around nylon fibres coated in Concanavalin A, a lectin with similar carbohydrate specificity. Mucilage, produced when *T. harzianum* is in close contact with *S. rolfsii*, may also be involved in the recognition process (Benhamou and Chet 1996).

Polysaccharide hydrolases are triggered early after recognition events. *T. harzianum* demonstrated high β-1,3-glucanase activity when cultured on cell walls and sclerotia of *S. rolfsii* (Elad *et al.* 1982). The mycoparasitic interaction prompts changes in the activity of chitinases of both host and mycoparasite (Inbar and Chet 1995). Before contact with the parasite, *S. rolfsii* exhibited high 1,4-β-*N*-acetylglucosaminidase (M_r 116 kDa) activity, while *T. harzianum* displayed low, constitutive activity of a 102 kDa chitinase (CHIT102). Post contact between host and parasite, N-acetylglucosaminidase activity of *S. rolfsii* ceased while CHIT102 activity was greatly elevated in *T. harzianum*. The CHIT102 activity of *T. harzianum* was substantially reduced later in the interaction and two different chitinases were produced: a 1,4-β-*N*-acetylglucosaminidase (CHIT73) and an endochitinase (CHIT50) which displayed high and low activities respectively. The addition of cycloheximide inhibited the production of chitinases, indicating that synthesis was *de novo*. Induction of the chitinolytic system occurred in the presence of living *S. rolfsii* mycelium or when in contact with lectin-coated nylon fibres and not by nylon fibres or lectins individually. Thus the events triggered during recognition are instrumental in the

switch to mycoparasitism. It has been suggested that proteases secreted by the host may have an important role in stimulating the chitinolytic system of *T. harzianum* (Inbar and Chet 1995). Such proteases could be responsible for degrading CHIT102 and activating CHIT73; the proteases may be constitutively synthesised or induced and released in response to recognition events.

The obvious activities of cell wall-degrading enzymes produced by *T. harzianum* in direct response to contact with *S. rolfsii* would suggest an exclusive mycoparasitic relationship, supported by the penetration of host cells by the parasite. However, ultrastructural studies exposed a more complex interaction involving the production of toxins to predispose the host (Benhamou and Chet 1996). Sclerotia were parasitised by *T. harzianum*, which colonised and penetrated the inner and outer layers of the rind and extensive cell alterations were evident in advance of penetration. In addition, *T. harzianum* switched mode of growth from intracellular when colonising the rind of the sclerotia to intercellular as it progressed into the medullary region. The parasite appeared to obtain nutrients from penetration of rind cells, by means of constricted hyphae that ramified abundantly. Partial degradation of the fibrillar matrix of the medulla could supply the parasite with nutrients during its intercellular growth. Colonisation of the medullary layer coincided with several cell alterations: retraction and aggregation of cytoplasm and vacuole breakdown. Toxic diffusible metabolites could be responsible for such predisposition (Benhamou and Chet 1996).

The integrity of the host cell wall was not affected by parasitism, even at penetration sites (Benhamou and Chet 1996). Labelling with WGA/ovomucoid-gold, gold-complexed β-1,3-glucanase and gold-complexed lipoprotein lipase all revealed regular distribution of relevant cell wall monomers. These insights suggest that cell wall depolymerases are not the first event in the antagonism of sclerotia of *S. rolfsii* and the initial phase could involve antifungal compounds during an antibiosis interaction. While hydrolytic enzymes have an important role in host cell wall penetration, it possible that continued secretion contributes to a saprophytic-like stage vital for rapid cell content utilization (Benhamou and Chet 1996). Therefore *T. harzianum* antagonism of *S. rolfsii* could involve several consecutive mechanisms: antibiosis, followed by mycoparasitism and finally competitive saprophytic ability.

Evidence for several different mechanisms involved in one antagonistic interaction is growing (Schirmbock *et al.* 1994; Belanger *et al.* 1995; Lorito *et al.* 1996). However, much of the determination of "a mechanism" appears to depend on the definitions used for the particular system studied. For example, Jeffries (1997) considers competitive antagonism and parasitism to involve one or more of the following: antibiotic production, secretion of lytic enzymes, hyphal interference and direct penetration of the host. There would appear to be significant overlap and the only difference between the two types of antagonism must be ascertained by defining the resource involved. The inhibition of a fungus by the secretion of antifungal compounds is also defined as 'antibiosis' (see below). Sometimes, therefore, it may be appropriate to consider an interaction under the general term of 'antagonism'. The antagonism of *Botrytis cinerea* by *T. harzianum* is such a case. The presence of the host induces coordinated formation of hydrolytic enzymes and peptaibol antibiotics (Schirmbock *et al.* 1994). The consequence of this parallel formation was discovered when the enzymes and antibiotics were assayed, individually, for ability to inhibit host spore germination and hyphal elongation. While individually these mycoparasitism-related proteins inhibited the host fungus, the synergistic effect of combined proteins was significantly higher. Lorito *et al.* (1996a) reported that peptaibols inhibited *B. cinerea* β-glucan synthase, thus preventing re-synthesis of the host cell wall and enhancing the degradative action of β-1,3-glucanases.

An important similarity between the antagonism of *R. solani* and *S. rolfsii* is the role of the recognition process. Lectin-carbohydrate interactions appear to trigger a cascade of antagonistic events. The recognition event appears to stimulate coordinated induction of synergistic hydrolytic enzymes and toxic proteins and thus represents the switch to mycoparasitism. The regulation of these mycoparasitism-related proteins is also controlled by glucose repression. Induction and repression are regulated at the transcriptional level, since *T. harzianum* cultures in the presence of cell walls of either pathogen revealed a rapid increase in mycoparasitism-related mRNA (Geremia *et al.* 1993; Vasseur *et al.* 1995). Therefore it is suggested that a common induction pathway with a specific inducer, perhaps chitin monomers or oligomers, is responsible for the expression of all mycoparasitic-related hydrolytic enzymes (Geremia *et al.* 1993).

Mycoparasitism can be extremely complex involving varied mechanisms depending on the stage of the antagonistic interaction and the environmental conditions. Laboratory and field investigations help to reveal the major strategies utilized by a certain parasite but variations and different capabilities should be expected within the field.

Additional mechanisms of interactions

Antibiosis is the term for another antagonistic interaction, a definition of which follows "those interactions that involve a low molecular weight diffusible compound or an antibiotic produced by a microorganism, that inhibits the growth of another microorganism" (Handelsman and Parke 1989). This mechanism involves antibiotics, either volatile or non-volatile that are fungistatic or fungicidal. The literature also reports accumulating evidence for the role of enzymes (Fravel, 1988) at various stages of antibiosis. This resulted in an expanded definition "inhibition or destruction of an organism by the metabolic production of another" (Baker and Griffin 1995). Hence antibiotics, lytic enzymes, small toxic molecules and volatiles should be included. The distinction between antibiosis and parasitism and perhaps even competition is slight with this later definition.

Cooke and Rayner (1984) proposed that mycelial interactions are competitive, neutralistic or mutualistic. "Competitive" interactions are detrimental to either or both; "neutralistic" are neither detrimental nor beneficial to both competitors; "mutualistic" interactions result in benefits for both organisms. Environmental fluctuations may change the direction of the benefit in such an association. The significance of neutralism and mutualism is unknown however, it is thought to be widespread and reduce competition, thus allowing fungi to coexist (Cooke and Rayner 1984).

1.3 Cell wall-degrading enzymes of *Trichoderma harzianum*

Trichoderma harzianum secretes many cell wall-depolymerising enzymes, frequently as a direct response to recognition events within the environment. Extracellular enzymes are thought play important roles in the antagonism of other fungi (Benhamou and Chet 1993). The battery of hydrolytic enzymes secreted by *T. harzianum* may offer an explanation as to survival of such fungi in the highly competitive environment of the phyllosphere and rhizosphere (Lorito *et al.* 1998). The aim of this section is to consider the different enzymes produced by *T. harzianum* and their relationship with the possible resources

available in mushroom cultivation. The resources include the cell walls of *A. bisporus* and wheat straw.

The activities of many *T. harzianum* enzymes have been studied, particularly with regard to the potential of this fungus in the biocontrol of several phytopathogens (Elad *et al.* 1982, 1984; Geremia *et al.* 1993; Carsolio *et al.* 1994; Thrane *et al.* 1997). Some *T. harzianum* depolymerases appear to have inhibitory effects when acting alone; others work in synergy to enhance the effect. Some enzymes are exceptionally potent as demonstrated by the tobacco and potato plant transformations to express a *T. harzianum* endochitinase gene (*ThEn-42*) which resulted in high tolerance or complete resistance to several phytopathogens (Lorito *et al.* 1998).

Therefore, with consideration to the relevant nutrient resources available to *T. harzianum* when colonising mushroom compost, the following hydrolytic enzymes will be discussed: chitinases, glucanases, cellulases, xylanases and protease.

1.3.1 Proteases of T. harzianum

The secretion of proteases by *T. harzianum* in conditions of simulated mycoparasitism has been reported (Elad *et al.* 1982; Ridout *et al.* 1988) and roles in the degradation of host cell walls, plasma membranes and cell contents were suggested (Geremia *et al.* 1993). There could also be a role for proteases in activating secreted proenzymes. A serine proteinase (Prb1) has received much attention, as described above, for its part in the mycoparasitism of *R. solani* (Geremia *et al.* 1993; Flores *et al.* 1997). Prb1 is induced by chitin and *R. solani* cell walls and is regulated at transcription level; the enzyme is repressed by glucose. As a classical serine proteinase, Prb1 was totally inhibited by phenylmethylsulfonyl fluoride (PMSF) and it was substrate specific for the chymotrypsin substrate Succ-Ala-Ala-Pro-Phe-pNA. Geremia *et al.* (1993) reported that Prb1 protein sequence exhibited high homology with subtilisin-like proteinases. The *prb1* gene is present as a single copy (Geremia *et al.* 1993) and has been assigned to chromosome VI in *T. harzianum* (Herrera-Estrella *et al.* 1993). The importance of this enzyme was illustrated when up-regulation of the gene resulted in significantly increased control of *R. solani* (Flores *et al.* 1997).

1.3.2 Beta-glucanases of Trichoderma species

One of the major structural polysaccharides of fungal cell walls is β-1,3-glucan and therefore β-1,3-glucanase activity in mycoparasitism is highly significant. A complex system of both endo- and exo-β-1,3-glucanases has been discovered for *T. harzianum* (de la Cruz *et al.* 1995; Benhamou and Chet 1997; Vazquez-Garciduenas *et al.* 1998). A recent study proposes some seven β-1,3-glucanases comprise the β-1,3-glucanolytic system of *T. harzianum* (Vazquea-Garciduenas *et al.* 1998). Certain β-1,3-glucanases appear to be constitutively expressed (de la Cruz *et al.* 1993; Vazquez-Garciduenas *et al.* 1998). Those β-1,3-glucanases apparently linked to mycoparasitism are repressed by glucose and induced by β-1,3-glucan-containing polysaccharides (Vazquez-Garciduenas *et al.* 1998) and sometimes, by chitin (Lorito *et al.* 1994; de la Cruz *et al.* 1995).

In the mycoparasitism of *Pythium ultimum*, a β-1,3-glucanase secreted by *T. harzianum* has an important role (Thrane *et al.* 1997). This enzyme alone was capable of inhibiting elongation of *P. ultimum* germ tubes and an even greater synergistic effect was achieved in combination with a fungicide. Benhamou and Chet (1997) indirectly demonstrated high β-1,3-glucanase activity as gold-labelled β-1,3-glucans in *P. ultimum* cell walls disappeared rapidly when antagonised by *T. harzianum*.

T. harzianum secretes a novel β-1,3-glucanase (BGN13.1) in the presence of fungal cell wall polymers or autoclaved mycelia of *B. cinerea*, *R. solani*, *Phytophthora citrophthora* and cells of *Saccharomyces cerevisiae* (de la Cruz *et al.* 1995a). BGN13.1 displays activity specific for β-1,3-linkages and has been determined as endolytic since δ-gluconolactone (inhibitor of exo-acting glucanases) has no effect on its activity. Induction of and glucose repression of this enzyme is controlled at the transcriptional level. The protein consists of three regions: an N-terminal leader sequence, a non-defined sequence and a sequence rich in cysteine at the C-terminal, have been found from amino acid sequence (de la Cruz *et al.* 1995a). Such Cys-rich motifs have been found in proteins capable of binding to plasmalemma components. Therefore, it is possible that the Cys-rich region may enable BGN13.1 to interact with non-substrate polysaccharides. This β-1,3-glucanase is thought to represent a new class of glucanases as it lacks conserved regions, contains a Cys-rich region and exhibits no cross reaction with antiserum raised to other glucanases. BGN13.1 alone is incapable of producing hydrolytic halos on medium containing purified fungal cell

walls. However, when combined with β-1,6-glucanase or chitinase, the enzymes work in synergy to degrade fungal cell walls. De la Cruz *et al.* (1995) suggested a possible role for BGN13.1 in the first steps of mycoparasitism since it is expressed along with chitinases, β-1,6-glucanases and proteases.

An endolytic β-1,3-glucanase, induced by chitin or fungal cell walls has been characterized (Noronha and Ulhoa 1996). Inhibition studies revealed the possible importance of sulfhydryl groups since Hg^{2+} ions completely inhibited enzymic activity. However, the enzyme was slightly stimulated in the presence of Zn^{2+} and Ca^{2+}, suggesting the requirement of metal ions for catalytic activity.

Two-dimensional gel electrophoresis revealed at least six β-1,3-glucanases: two with molecular mass of 77 kDa, three at 60 kDa and one smaller form at 35 kDa (Vazquez-Garciduenas *et al.* 1998). These β-1,3-glucanases, induced by simulated mycoparasitic conditions, could be triggered by two host cell wall components. It was suggested that both an extractable cell wall component and a cell wall-bound element were capable of inducing transcription of the β-1,3-glucanases genes (Vazquez-Garciduenas *et al.* 1998).

Trichoderma longibrachiatum produced laminarinases (β-1,3(4)-glucanase) when cultured in medium containing ground *A. bisporus* fruiting bodies (Sharma and Nakas 1987). This activity was inhibited by Mn^{2+}, Hg^{2+}, N-bromosuccinimide and potassium permanganate. The effect of mercury ions and N-bromosuccinimide also suggested the involvement of sulfhydryl groups for enzyme activity. Laminarinases of *T. longibrachiatum* were glucose-repressed; in the presence of D-glucose, only constitutive laminarinases were expressed (Tangarone *et al.* 1989).

1.3.3 Production of chitinases by T.harzianum

Evidence for a role in mycoparasitism for chitinases is quite extensive: i) chitinases are expressed in cultures before or just after contact with host (Carsolio *et al.* 1994; Inbar and Chet 1995), ii) pure chitinases inhibit several fungi (Di Pietro *et al.* 1993; Lorito *et al.* 1993, 1994), iii) chitinases are induced by autoclaved host mycelia in mycoparasitic *T. harzianum* but not in non-mycoparasitic strains (Garcia *et al.* 1994) and iv) an increase in the activity of endochitinase in *T. harzianum*-treated rhizosphere resulted in increased

levels of biocontrol (Dal Soglio *et al.* 1998). Differences are apparent between chitinolytic systems of *T. harzianum* strains and these could be responsible for the different abilities of these strains to control various phytopathogens. The chitinolytic systems of *T. harzianum* consist of chitinases [poly(1,4-(*N*-acetyl-*β*-D-glucosaminide))glucanohydrolase] and chitobiase (*N*-acetyl-*β*-glucosaminidase) (Ulhoa and Peberdy 1991). Chitinolytic activity may be in endo- (random, internal cleavage) or exo- (cleavage from non-reducing end of polymer) fashion in cleavage of the chitin polymer (β-1,4-linked polymer of *N*-acetylglucosamine).

De la Cruz *et al.* (1992) described three chitinases induced by chitin and chitin-containing fungal cell walls, that were also repressed by glucose: 42 kDa (CHIT42), 37 kDa (CHIT37) and 33 kDa (CHIT33). All three enzymes exhibited endolytic activity and were synthesised *de novo*; chitinase activity increased in parallel to an increase in protein appearance. CHIT42 was capable of lysing *B. cinerea* cell walls and although CHIT37 and CHIT33 were incapable of such lytic activity individually, they had a positive synergistic effect on the activity of CHIT42. Antifungal activity was not achieved with CHIT42 alone and therefore supports the hypothesis that a complement of depolymerases is necessary for the effective degradation of host cell walls.

A chitobiase and an endo-chitinase were produced by *T. harzianum* strain P1, capable of inhibiting germination and germ tube elongation of *B. cinerea, Fusarium solani, Ustilago avenae, Uncinula necator, T. harzianum* strain 22 and *Saccharomyces cerevisae* (Lorito *et al.* 1993). There was a substantial synergistic effect on inhibition when the chitinase and chitobiase were combined and this effect was increased by the addition of β-1,3-glucanase. Chitinases were capable of attacking all stages of fungal growth on host fungi and *T. harzianum* P1 displayed resistance to its own chitinase activity, which may be due to production of a specific inhibitor or because of cell wall alterations that protect the chitin content (Lorito *et al.* 1993).

Cloning of genes has allowed characterization at the molecular level and several researchers have concentrated on the major endochitinase, Ech42 (CHIT42) (Hayes *et al.* 1994; Carsolio *et al.* 1994, 1999; Garcia *et al.* 1994; Lorito *et al.* 1996b; Woo *et al.* 1999). The gene *ech42* contains an open reading frame (ORF) encoding a protein of 424 amino

acids (aa) (Hayes *et al.* 1994). The gene is present as a single copy and has been assigned to chromosome V or VI (Carsolio *et al.* 1994). *Ech42* contains two highly conserved regions, a putative catalytic activity region and leader sequence, exhibited by other endochitinases from *Serratia marcescens, Bacillus circulans* and *Vibrio parahemolyticus* (Hayes *et al.* 1994). The putative protein encoded by the gene predicts a molecular mass of 46 kDa, somewhat larger than the mature, processed protein (42 kDa) and this is due to a secretion sequence and a processing site (Carsolio *et al.* 1994; Hayes *et al.* 1994; Garcia *et al.* 1994). The processing sequence displays homology to sites for the Kex 2 proteinase, which could be involved in activating Ech42. *B. cinerea* and *R. solani* induced the expression of *ech42* since mRNA was not detected when *T. harzianum* was cultured in glucose-containing medium. *Ech42* was also induced by light-induced sporulation (Carsolio *et al.* 1994). Expression following light-induced sporulation could be controlled by a specific regulatory protein, as a BrlA binding box sequence was discovered in the promoter of *ech42*.

CHIT33, an endochitinase, displays high homology with fungal and plant chitinases, particularly with the essential residue in the active site (Limon *et al.* 1995). The gene encodes a 321 aa protein; the first 19 residues represent a putative signal peptide for secretion of the protein. The enzyme is repressed by glucose and induced under conditions of starvation. CHIT33 lacks three domains characteristic of chitinases: i) Ser/Thr-rich domain, ii) chitin-binding domain and iii) C-terminal processing. The absence of these domains suggests that the enzyme is not associated with morphogenesis but instead has a nutritional role, be it saprophytic or mycoparasitic (Limon *et al.* 1995).

An exochitinase has recently been purified from *T. harzianum* which is smaller (28 kDa) than other chitinases described in the chitinolytic system (Deane *et al.* 1998). The exochitinase is induced by chitin and repressed by glucose. The activity of the enzyme is specific for substrates of more than two units of *N*-acetylglucosamine. This enzyme is glycosylated with glucosamine, mannose and galactose, which can be common in exo-type enzymes (Deane *et al.* 1998). This is the only known *T. harzianum* exochitinase capable of degrading insoluble chitin substrates (Deane *et al.* 1998).

Haran *et al.* (1995) separated chitinases three classes: i) β-1,4-*N*-acetylglucosaminidases split diacetylchitobiose, chitotriose, chitotetrose in an exo-type manner releasing *N*-acetylglucosamine; ii) endochitinases, which cleave random, internal sites in the chitin polymer releasing low molecular weight oligomers and iii) exochitinases (chitobiosidase), cleave diacetylchitobiose (but not monomers) in an exo- manner. The chitinolytic system was described according to the molecular mass of the chitinases: CHIT102, CHIT73 (both β-1,4-*N*-acetylglucosaminidases), CHIT52, CHIT42, CHIT33 and CHIT31 (all endochitinases) (Haran *et al.* 1995). All six enzymes were induced by chitin and CHIT102 exhibited low levels of constitutive activity. The full complement of six chitinases was suggested to be required for maximum efficiency against a broad range of hosts.

Synthesis of chitinases with a putative role in mycoparasitism appear to be regulated by an induction-repression cycle; some chitinases differ in the induction mechanism. Ulhoa and Peberdy (1991) proposed that chitinases were induced by chitin-containing media and repressed by glucose and *N*-acetylglucosamine (chitin monomers). Initially chitobiose oligomers were thought to induce the chitinolytic system, however these fragments are rapidly degraded by constitutive chitobiase, the hydrolysis product of which would result in catabolic repression. Therefore other constitutive chitinases are suggested to produce different soluble chitin oligomers capable of inducting mycoparasitism chitinases. While CHIT42 is induced by the presence of chitin or chitin-containing fungal cell walls, CHIT33 activity is stimulated by glucose de-repression providing evidence of two regulation mechanisms (Limon *et al.* 1995).

In some cases of mycoparasitism, where the action of depolymerases is a very early event, the recognition reaction is suggested to trigger the induction of chitinases (Inbar and Chet 1995). During a mycoparasitic interaction between *T. harzianum* and *S. rolfsii,* constitutive acetylglucosaminidase activity was observed before contact. Subsequently marked changes occurred in chitinase expression and activities post contact (Inbar and Chet 1995; Haran *et al.* 1996). *S. rolfsii* exhibited no chitinase activity post contact with *T. harzianum.* The constitutive activity of *T. harzianum* (CHIT102) increased after contact and subsequently decreased and was replaced by CHIT73 and CHIT50 activity. The induction of these mycoparasitism-related chitinases could, therefore be linked to the recognition events

which require a living host since CHIT102 alone was induced on medium containing autoclaved *S. rolfsii* mycelium (Inbar and Chet 1995).

Differential expression of chitinases may be responsible for the specific host range of a particular strain of *T. harzianum* (Chet and Inbar 1995; Haran *et al.* 1996). In interactions with *R. solani* β-1,4-*N*-acetylglucosaminidase is initially induced followed by several endochitinases. *S. rolfsii* triggers β-1,4-*N*-acetylglucosaminidase activity only, by *T. harzianum* (Haran *et al.* 1996). It is possible that the recognition of these two phytopathogens dictates the chitinases induced and hence, the antagonistic ability of the strain of *T. harzianum*.

The endochitinase Ech42 appears to be under the control of a carbon catabolite repressor protein (Cre1) during interactions between *T. harzianum* and *B. cinerea* (Lorito *et al.* 1996b). The promoter sequence for *ech42* contains two binding sites for Cre1. The Cre1 complex appears to bind to DNA of non-mycoparasitic mycelia for which it has high affinity. Under mycoparasitic conditions a mycoparasitic protein-DNA complex out-competes Cre1. The model proposed suggests that the binding sites for Cre1 and mycoparasitic complex (RSB) are either overlapping or contiguous. Therefore under non-mycoparasitism conditions Cre1 protein has a higher affinity for its target sequence to which it binds, precluding the binding of the smaller RSB. Once contact between the host and parasite is established Cre1, now functionally impaired, dissociates from its binding site allowing the RSB to bind and induce gene expression.

Several genetic manipulations have investigated the roles of chitinases during mycoparasitism (Margolles-Clark *et al.* 1996a, 1996b; Carsolio *et al.* 1999; Woo *et al.* 1999). The level of secretion of the major endochitinase Ech42 has been altered. To increase Ech42 production, *T. reesei* was transformed to express *ech42* under the cellulase promoter *cbh1* (Margolles-Clark *et al.* 1996a). Multiple gene copies were inserted into the *T. reesei* genome and a twenty-fold increase in Ech42 activity was recorded, compared to *T. harzianum*.

Under control of *cbh1* promoter Margolles-Clark *et al.* (1996b) reported a five-fold increase in extracellular production of Ech42 and a ten-fold increase in Ech42 activity. The

marked increase in activity was due to a synergistic effect since other hydrolytic enzymes were secreted. The difference in activity between the *T. harzianum* and *T. reesei* transformants could be assigned to the fact that *cbh1* requires induction to be functional in *T. harzianum*.

Trichoderma harzianum transgenics have also been produced which contain multiple copies of *ech42* or disrupted *ech42* (Carsolio *et al.* 1999; Woo *et al.* 1999). The mutants designed to overexpress Ech42 (QL) exhibited a 42-fold increase in activity, under inducing conditions, compared to the wild type (WT). Disruption mutants (ΔQ) produced little or no Ech42 activity (Carsolio *et al.* 1999). Western blots revealed no chitinase protein for ΔQ, but QL displayed a signal six times stronger than WT. During confrontation assays between *T. harzianum* and *R. solani*, QL transgenics inflicted greater cell wall alterations than WT and ΔQ mutants at the same time point. However, 16 hours after contact the host cell wall had been degraded by all strains of *T. harzianum* (Carsolio *et al.* 1999). The basal expression of QL transgenics was higher than the WT and this is suggested to be responsible for the initial cell wall degradation. In glasshouse experiments with WT *T. harzianum,* disease incidences of 33% and 25% for *R. solani* and *S. rolfsii*, respectively were achieved (*cf.* 50 % and 65% disease respectively, in the absence of *T. harzianum*). Treatment with a ΔQ transgenic achieved very similar levels of disease control, while QL mutants exhibited greater disease reductions against *R. solani* but no difference to the WT against *S. rolfsii* (Carsolio *et al.* 1999). Thus overexpression of Ech42 had no effect on *S. rolfsii* and therefore it does not appear to have a vital role in mycoparasitism (Haran *et al.* 1996; Carsolio *et al.* 1999). The decrease in disease incidence with QL *T. harzianum* was much less proportionately than the increase in enzyme activity. This evidence supports the proposal that a combination of chitinases is required for efficient cell wall degradation; the synergistic effect is probably greater than that of increased activity of one enzyme. Deletion mutants continued to degrade host cell walls and therefore the deletion of one protein was compensated for by the induction of other chitinases (Carsolio *et al.* 1999).

The role of Ech42 was also studied in mycoparasitism of *B. cinerea* using gene disruption (Woo *et al.* 1999). Mutants with the disrupted *ech42* lacked mRNA transcripts, the protein and Ech42 enzyme activity, but all other chitinolytic and glucanolytic enzymes were

expressed normally. Ech42 appeared to be essential for parasitism of *B. cinerea* since the addition of Ech42 to the level secreted by the WT restored the biocontrol capability of the mutant. Although the mutant *T. harzianum* had no effect towards *R. solani* on bean leaves, when added to *R. solani* the mutant increased plant survival (16%) and enhanced plant growth when compared to the WT strain. This provides evidence that there are two different mechanisms employed by *T. harzianum* strain P1 in the antagonism of *B. cinerea* and *R. solani*. The improved control of *R. solani* by D11 appears to correlate with increased rhizosphere competence of the mutant (Woo *et al.* 1999). D11 demonstrated reduced antifungal activity towards *R. solani*, but the mutant grew more abundantly on plant seeds than the WT and is therefore may have excluded *R. solani* from the spermosphere and rhizosphere. The disruption method again revealed alternative mechanisms for successful antagonism if key enzymes are eliminated.

Genetic manipulations can elucidate specific roles for the various hydrolytic enzymes but also emphasize the complexity of such mechanisms. These studies also reveal secondary mechanisms that may be employed simultaneously or as a 'back-up' if the original mechanism fails.

1.3.4 Production of cellulases by Trichoderma spp

The cellulases are considered in this section with relevance to possible saprophytic capabilities of *T. harzianum* in mushroom compost. Cellulose is a linear β-1,4-glucosidically-linked homopolymer, which is insoluble in its crystalline form. Cellulose polymers associate into highly ordered fibrillar structures. These fibrils are physically complex with both crystalline and amorphous regions. This polysaccharide is the major structural component of plant cell walls and by nature of it complex structure, is not readily amenable to enzymatic degradation. Cellulose is also a structural component of some fungal cell walls, specifically the Oomycetes and Hyphochytridiomycetes (Bartnicki-Garcia 1968).

Successful saprophytic fungi utilize cellulose in plant debris as a source of nutrients; this is termed 'cellulolysis'. However in order to achieve effective cellulolysis, fungi must produce a range of cellulases with different modes of action. There are three types of cellulase, all of which degrade the same β-1,4-glucosidic bond although they act on different parts of the cellulose molecule. All three cellulases are required for efficient

degradation of crystalline cellulose. The three types of hydrolytic activity are: i) endo-β-1,4-glucanases (EC 3.2.1.4) which cleave internal β-1,4-glucosidic bonds, ii) exo-β-1,4-glucanases or cellobiohydrolases (EC 3.2.1.91) which release cellobiose, a disaccharide, from the ends of cellulose chains, and iii) β-1,4-D-glucosidases (cellobiase EC 3.2.1.21) which subsequently hydrolyze cellobiose and other short oligosaccharides to glucose (reviewed by Goyal *et al.* 1991). Oxidative enzymes (cellobiose quinone oxido-reductase and cellobiose oxidase) could also have roles in cellulolysis. Rather than a degradative role, various oxidases are suggested to be involved in the regulation of cellulase synthesis (reviewed by Goyal *et al.* 1991). There are two comprehensive reviews on cellulolysis (Wood and Campayo-Garcia 1990; Goyal *et al.* 1991) which have been used in the basic composition of this section.

The classical hypothesis on hydrolytic action of the cellulases is thought to involve an initial attack by endoglucanases (EG) at random, internal sites in soluble cellulose or amorphous regions of cellulose microfibrils that create more active sites for the exo-acting cellobiohydrolases (CBH). Subsequent synergistic action of EGs and CBHs produce small oligosaccharides, substrates for β-glucosidase (Goyal *et al.* 1991). Through this action, cellulose is degraded to glucose and is readily assimilated by the fungus.

All fungal cellulolytic systems studied so far comprise multiple enzyme components (Wood and Garcia-Campayo 1990). The cellulase system of *T. reesei* consists of six endoglucanases (EG I-VI), three cellobiohydrolases (CBH I-III) and one β-glucosidase (reviewed by Goyal *et al.* 1991). Multiple components could be products of multiple genes or artefacts of the procedures used in purification or result of proteolysis. Since there are few fungal cellulase genes multiple forms are probably artefactual (e.g. complexes with Ampholyte carrier proteins) (Farkas *et al.* 1985) or a product of biosynthetic routes i.e. assembled from precursors with different cellulase activity (Goyal *et al.* 1991). Proteolysis has been suggested as a direct source of multiple enzymes. Cleavage of CBHI yielded a catalytic domain and a binding domain and changed the activity of the enzyme. The catalytic core was not active towards insoluble cellulose but its hydrolytic activity on soluble cellulose was unaffected (Van Tilbergh *et al.* 1986). Thus post-translational modification may have an important role in the production of multiple enzymes (Goyal *et al.* 1991). Messner and Kubicek (1988) provided direct evidence of the production of low

molecular weight precursors preceding EG secretion. EGs were suggested to be the product of precusors with *O*-glycosidic links between sugar units (as EGs treated with α-mannosidase reverted to low molecular weight proteins identical in size to precursors). Furthermore interference with *O*-glycosylation inhibited EG secretion, in contrast secretion of EGs was not affected by Endo H treatment suggesting that *N*-glycosylation may not be involved in secretion (Messner and Kubicek 1988).

Bacterial and fungal cellulases share a common tripartite structure: a flexible hinge region links the catalytic domain (non-conserved) and the conserved cellulose-binding domain (CBD) (Wood and Campayo-Garcia 1990). The hinge region and CBD of *T. reesei* cellulases share high homology although the position of the CBD may vary i.e. C-terminus in CBHI and EGI and N-terminus for CBHII and EGIII. *T. reesei* cellulases exhibit 70% homology for CBDs and consist of 30 aa. The hinge region is rich in glycine and cysteine and is stabilized by 2 or 3 disulphide bridges (Bhikhabai and Pettersson 1984). The binding domain is a small region separated from the catalytic core and comprises a large ellipsoid head with a long tail (Schmuck and Pilz 1986). The linker region shows lower homology (50-60%) between *T. reesei* cellulases and is rich in proline, serine and threonine. This region is heavily *O*-glycosylated, which has been suggested to protect against proteolysis (Wood and Campayo-Garcia 1990). The presence of the CBD improves binding of cellulases onto insoluble substrates but has no effect on enzymic activity against soluble substrates (Srisodsuk *et al.* 1997). Binding is dependent on a precise three-dimensional structure and this has been further described as wedge-shaped (Teeri *et al.* 1990) with a hydrophilic and hydrophobic surface. Srisodsuk *et al.* (1997) replaced the CBD of CBHI with that of EGI and the hybrid enzyme exhibited greater affinity for cellulose, which was similar to that of EGI.

The non-conserved catalytic domains differ most in the shape of the active site; EGs have an open, cleft-like active site and CBHs produce a tunnel-shaped active site (Penttila *et al.* 1986). The catalysis of cellulose is thought to occur by an acid hydrolysis mechanism (Penttila *et al.* 1986). This argument is based on the catalytic site of hen egg white lysozyme, in which residues Glu-35 and Asp-52 act as proton donor and acceptor, respectively. EGI also has Glu and Asp residues in similar positions to CBHI (Penttila *et al.* 1986). *T. reesei* cellobiohydrolases have different stereospecificities. CBHI acts at the

reducing end of a cellulose polymer, while CBHII attacks the non-reducing end (Teeri *et al.* 1998).

T. harzianum is also recognised for secreting cellulases and this is illustrated in a mycoparasitic relationship with *Pythium ultimum*, an Oomycete (Thrane *et al.* 1997). Contact with the host *P. ultimum* induced the secretion of an endo-β-1,3-glucanase and two cellulases. The purified *T. harzianum* enzymes were capable of inhibiting germination and germ tube elongation of *P. ultimum* and the three components of cellulase activity from *T. harzianum* were recognised.

In cultures containing 2% wheat straw as sole carbon source *T. harzianum* produced several isoforms of β-glucosidase (Todorovic *et al.* 1990). All isoforms exhibited cellobiase and 4-nitrophenyl *β*-D-glucosidase (4NPGase) activity and were inhibited by glucose. In addition, 4NPGase activity was inhibited by cellobiose and D-glucono-1,5-lactone. The origin of these isocomponents was suggested to be the result of post-translational modifications such as proteolysis (Todorovic *et al.* 1990).

T. reesei secretes high quantities of protein of which 60% is accounted for by CBHI (Goyal *et al.* 1991). The production and secretion of large amounts of enzymes prompted interest from the commercial world and it is a direct consequence that much research has concentrated on the cellulases of *T. reesei*. The genes of most of the major cellulases have now been cloned and characterised.

Cellobiohydrolase I (CBHI) is the dominant secreted cellulase produced by *T. reesei*. It has a molecular mass of 42-72 kDa dependent on the level of glycosylation; carbohydrate content varies from 1.4-10.4% (Goyal *et al.* 1991). A protein of 496 aa and 52 kDa was predicted from the gene sequence (Shoemaker *et al.* 1983; Teeri *et al.* 1983). Endoglucanase I (EGI) is produced as a 459 aa preprotein of which, 22 residues represent a signal peptide to direct secretion of the enzyme. The signal peptide shares 45% homology with that of CBHI (Penttila *et al.* 1986). The mature protein comprises 437 aa and has a molecular mass of 46 kDa. Cellobiohydrolase II (CBHII) displays no homology to CBHI or EGI except in the binding region. The CBD (A) and hinge (B) that together produce the binding region are found at the N-terminus in CBHII. In addition, CBHII has two B

regions (B, B') both of which are heavily *O*-glycosylated (Teeri *et al.* 1987). Endoglucanase III (EGIII) gene is very different from the other 3 cellulases of *T. reesei*. In fact the only similarity with the other cellulases is the conserved binding region situated, like CBHII, at the N-terminus (Saloheimo *et al.* 1988). The activities of EGIII are unique and in part resemble EGI and are also similar to CBHII.

Endoglucanases cannot attack crystalline cellulose but will act synergistically towards it in the presence of CBHI and CBHII. The advent of new purification techniques, revealed that exo-exo synergy only occurred in the ratio of 1:1 (CBHI:CBHII) with the addition of EG and no synergism occurred between pure CBHI and CBHII (Wood and Campayo-Garcia 1990). Synergistic action between EGI and CBHII was investigated by following degree of polymerization of cotton cellulose after treatment with EGI or CBHII or both (Kleman-Leyer *et al.* 1996). EGI, with its internal mode of cleavage, decreased the degree of polymerization by 18-fold and a weight loss of 34% was recorded. CBHII failed to depolymerize this substrate. Combinations of CBHII and EGI released more soluble reducing sugars; this was probably due to the increased activity of CBHII on the ends of EGI degradation products. Synergism was negated by the addition of cellobiose, which decreased the synergy due to end-product inhibition of CBHII. The addition of β-glucosidase further increased the synergistic effect (by the removal of cellobiose) and this demonstrated the necessity for three different types of cellulase (Kleman-Leyer *et al.* 1996).

Regulation of the cellulolytic system of *T. reesei* is very complex and still not fully understood. While a common regulation pathway would appear to be present, evident by the simultaneous expression of the cellulases, some may also be specifically regulated. The expression of *cbh1, cbh2, egl1, egl2* and *egl5* was co-ordinated under a range of different conditions (Ilmen *et al.* 1997). The highest level of expression was the *cbh1* transcript and the carbon source affected the level of gene expression. Solka floc cellulose and the disaccharide sophorose induced the greatest cellulase expression. Moderate cellulase expression occurred with cellobiose or lactose as the sole carbon source and glucose inhibited cellulase expression. There is a possible role for CreA-like regulatory proteins in the glucose repression of cellulase genes. Site-specific mutagenesis of the *cbh1* promoter revealed regions with similarities to the binding sites of CreA of *Aspergillus nidulans* are involved in the regulation of CBHI, specifically its glucose repression (Ilmen *et al.* 1996).

This indicates a common mechanism of induction and glucose repression involved in regulating cellulase expression. Additionally, Ilmen *et al.* (1996) observed derepression of cellulase expression after the depletion of glucose and without the addition of an inducer. Further investigations revealed that derepression was not simply a result of carbon or nitrogen depletion and some form of induction was involved. This particular trigger of expression is suggested to be linked to growing cultures and could be induced by oligosaccaride molecules released from the cell walls of the starving fungus (Ilmen *et al.* 1997). Another hypothesis focuses on the production of low levels of constitutive β-glucosidase which could, by transglycosylation, produce sophorose or another inducing sugar and hence induce the expression of cellulases once glucose has been consumed (Ilmen *et al.* 1997).

It is commonly recognised that a cellulase inducer has to be formed in order to trigger cellulase expression (Ilmen *et al.* 1997; Seiboth *et al.* 1997). The roles of CBHs and EGs in the formation of cellulase inducers were investigated using a series of deletion mutants for the genes: *cbh1, cbh2, egl1* and *egl2* (Seiboth *et al.* 1997). The deletion of *cbh2* or *egl2* prevented the expression of all other cellulase genes. However, the deletion of *cbh1* or *egl1* had no effect on the expression of the other cellulolytic genes, except that Δ*cbh1* mutant produced elevated levels of the *cbh2* transcript. A mutant containing deletion of *cbh1* and *cbh2* rendered the fungus incapable of growth on cellulose and devoid of EG transcripts. This effect could be overcome by the addition of sophorose (β-1,2-glucosyl-glucose), which is considered an inducer, resulting in the expression of EGs. However, sophorose was only detected in very low concentrations in culture fluids from *T. reesei* growing on cellulose (Kubicek 1993). CBHII and EGII appear to have a major role in the efficient formation of inducer from cellulose and their absence eliminates the cellulose attacking ability of *T. reesei*. However, as neither CBHII nor EGII exhibit transglycosylation activity, neither enzyme is capable of producing sophorose. Another hypothesis suggests that cellobiose represents an inducer of the cellulolytic system (Kubicek 1993). Cellobiose is the major end-product of the initial attack on cellulose. This disaccharide is soluble and its up-take by the cell is thought to trigger cellulase expression. The addition of excessive cellobiose results in end-product inhibition, however the slow addition of cellobiose results in cellulase production in similar amounts as recorded in the presence of cellulose (Kubicek 1993). Both cellobiohydrolases could therefore be responsible for the production of a cellobiose inducer. In other systems (e.g. *Verticillium albo-atrum* and *Fusarium*

oxysporum f. sp. *lycopersici*) cellobiose has been deduced as an inducer of cellulases (Cooper and Wood 1975).

1.3.5 Production of xylanases by Trichoderma spp

The production of xylanases is considered here with regards to the possible saprophytic growth of *T. harzianum* in mushroom compost. Xylan is classified as a hemicellulose, which are heteropolysaccharides comprising of many different monomers and linkages. The monomers found in hemicellulose are D-xylose, D-mannose, D-galactose, L-arabinose, D-glucuronic acid and D-glucose. Xylan and mannan constitute the largest proportions of hemicellulose. Xylan consists of a polymer of β-1,4-xylose with β-1,2 linked side chains of 4-O-methylglucuronic acid and β-1,3-linked chains of arabinose (reviewed by Sinsabaugh and Liptak 1997). Endo-β-1,4-xylanase randomly cleaves the xylan backbone and subsequent β-xylosidase action reduces xylobiose to D-xylose. In comparison to lignin and cellulose, hemicellulose is relatively small and this renders it less recalitrant (Sinsabaugh and Liptak 1997).

Hemicellulose is found in plant cell walls where it functions to cross-link cellulose microfibrils and infer strength (see earlier) and therefore would be present in the compost as wheat straw (*ca.* 23% dry weight; Fermor TR, HRI Wellesbourne, pers. comm.). Another supply of hemicellulose in mushroom compost is contained within the rye grain used to make mushroom spawn. Carbohydrates comprise the majority of dry matter in cereal grain, typically 77-87% (Kent 1983). Cellulose and hemicellulose are the main components of the cereal grain cell wall (Kent 1983).

Xylanases are of particular importance in the food and animal feed industries and in pulp and paper industry. Research has concentrated on the xylanases of *T. reesei* due to the high quantities secreted by this species. *T. reesei* produces two major xylanases (XYNI and XYNII) (Tenkanen *et al.* 1992) which are the products of two genes (Torronen *et al.* 1992). These two xylanases contribute 50% of the total xylanase activity of *T. reesei* and have been characterised by Tenkanen *et al.* (1992) and Torronen *et al.* (1992). XYNI has a pI of 5.5 and molecular weight of 19 kDa and XYNII, at 20 kDa, has a pI of 9.0. A preference was exhibited for polymeric substances over xylo-oligosaccharides. This preference decreased with a decrease in substitution.

XYNI promoter exhibited catabolic repression in the presence of glucose and was activated by exposure to xylan and xylose, while XYNII promoter displayed basal activity with glucose, which was enhanced by xylan, xylobiose, sophorose and cellobiose (Zeilinger *et al.* 1996). A catabolic repressor protein complex has been implicated. Xylanase activity was generally produced simultaneously with cellulase (Zeilinger *et al.* 1996). The two xylanases also exhibited differences in response to end-products; XYNI was expressed in the presence of xylose but not xylobiose and XYNII was expressed in the presence of xylobiose only (Zeilinger *et al.* 1996). Since XYNI was repressed in the presence of glucose it has been suggested that this enzyme is derepressed rather than induced by hemicellulose, however expression studies revealed that XYNI is induced and derepressed by two separate mechanisms (Mach *et al.* 1996).

The structure of XYNII comprises of 2 antiparallel β-sheets which twist to form a cleft, which is thought to contain a putative active site (Torronen *et al.* 1994). Two amino acids, Glu86 and Glu177, have been proposed as components of the active site and of these Glu177 displayed a change in structural conformation at different pH that could be indicative of a catalysis mechanism (Torronen *et al.* 1994). XYNI also comprises of 2 antiparallel β-sheets, however the active site residues are thought to be Glu75 and Glu164 (Torronen *et al.* 1995).

T. harzianum also exhibits substantial xylanase activity and xylanases of identical size to XYNII (20kDa) of *T. reesei* have been reported (Campbell *et al.* 1993). When compared with 20 kDa xylanases of *Bacillus circulans* there was 51% homology at the level of amino acid residues, however the main-chain structures revealed 89% homology (Campbell *et al.* 1993). The putative active site of *B. circulans* xylanase contained residues Glu78 and Glu172 and these were suggested to have an involvement in catalysis. These residues are similar to those reported for the *T. reesei* XYNI and XYNII active sites and therefore it is likely that *T. harzianum* xylanases possess similar catalysis mechanisms.

Chapter Two

Materials and Methods

2.0 Culture and Maintenance of Fungal Isolates

2.0.1 Preparation of conidial stock suspensions

Spore suspensions (1.0×10^7 spores ml^{-1}) in 0.04% Tween 80 were prepared from single-spored cultures of *T. harzianum* isolates. The source of isolates, their aggressiveness and their molecular groups are summarised in Table 2.1. Spores were dislodged in 0.04% Tween 80 and clarified by filtration through two layers of sterile muslin to remove hyphal fragments. The spore concentration was determined using a modified haemocytometer counting chamber (Weber Scientific International Ltd, Teddington, UK) and subsequently corrected (see above). Conidia from suspensions were then pelleted by centrifugation and resuspended in an equal volume of 20% glycerol. Aliquots of spore suspensions were flash frozen in liquid nitrogen and stored at -70°C. In addition, stocks of *T. harzianum* isolates were also prepared on slopes of malt extract agar (MEA) and stored under liquid paraffin at 4°C.

2.0.2 Preparation of conidia

Sporulating cultures of *T. harzianum* were routinely prepared from the stock suspensions. An aliquot (100 µl) was inoculated on MEA using a glass spreader and then incubated at 25°C in the absence of light. After 3 days the plate, covered with hyphal growth, was removed from the incubator and exposed to daylight to stimulate sporulation. Spore suspensions were produced using the method described above.

2.0.3 Preparation of A. bisporus hyphal stocks

Hyphal stocks were prepared by inoculating MEA with white strain, A12 spawn grain (supplied by Dr H. Grogan, HRI Wellesbourne). Thick MEA plates (35 ml) were used and the antibiotics, penicillin and chloramphenicol (Sigma, UK) were added to prevent the development of secondary colonisers, since the growth of *A. bisporus* colonies was slow. Plates were incubated at 25°C in the dark for 2 to 3 weeks. Colonised plates were stored at 4°C for up to a month, after sealing the plates with parafilm. Spawn grain was also stored in sterile containers at 4°C. *A. bisporus* inoculum was taken as mycelial plugs with a cork borer (3 mm diameter) from the advancing edge of the colony.

Antibiotic MEA contained: 2% w/v malt extract, 0.05g chloramphenicol per litre (in 10 ml 95% ethanol) and 1 ml penicillin stock (100 units ml^{-1}) per 10 ml of cooled medium.

Table 2.1. Isolates of *Trichoderma harzianum* in culture.

Isolate	Origin	Molecular Group(a)
Th1(c)	UK	Th1
T28JF	UK	Th1
Th1M	UK	Th1
TD15	UK	Th1
T7	UK	Th2
T7 (reduced mutant)	UK	Th2
T32	UK	Th2
Th3(05)	UK	Th2
KPNT	UK	Th2
Th2F	UK	Th2
Th2A	UK	Th2
TD7	UK	Th3
IMI 110150	UK	Th3
Th3c	UK	Th3
A006022	UK	Th3
RM10 casing	USA	Th4
RM10 manure	USA	Th4
BE	USA	Th4
N050	UK	*T. hamatum*
A111JF	UK	*T. hamatum*

(a) Describes the molecular group designated by Muthumeenakshi et al (1994); Th1 - non-aggressive colonisers of mushroom compost; Th2 - aggressive colonisers of compost, indigenous to the UK; Th3 - non-aggressive compost colonisers, some of which are not *T.harzianum*; Th4 - aggressive colonisers of compost, indigenous to USA and Canada. All isolates kindly supplied by HRI Wellesbourne.

2.1 Visual assessments and quantification of fungal growth in mushroom compost

2.1.0 Fungal inoculation of mushroom compost

A model of green mold disease in mushroom compost was prepared by the inoculation of *T. harzianum* and *A. bisporus* in a tube (60 ml, polypropylene) containing mushroom compost (Formula 8, kindly supplied by Mushroom Unit, HRI Wellesbourne). *A. bisporus* (strain A12) was inoculated on spawn grain. Generally three spawn grains were added to the bottom of the tube, 'adjacent' to the

inoculum however, in some experiments *A. bisporus* spawn was separated spatially from *T. harzianum* by placing spawn at the top of the tube (see Chapter Three for variations). Conidia of *T. harzianum* were inoculated by rolling spawn grain or rye grain in profusely sporulating cultures of *T. harzianum*. The *T. harzianum* inoculum was placed in the bottom of the tubes for all experiments. Fungal inocula were then covered with *ca.* 30 g of fresh mushroom compost, compressed by hand. The lids were tightened and then released a quarter turn to prevent the development of anaerobic conditions. Tubes were placed in a 25°C incubator with high relative humidity in the absence of light for 3 weeks.

2.1.1 Visual assessment of fungal growth

Fungal growth was determined using two scales. *A. bisporus* growth was measured on a scale of 0 (no visible growth) to 3 (full colonisation of tube). Initially, four tubes were chosen to represent the scores 0 to 3 and these were used to assign scores to all other tubes enabling a consistent assessment. The distance (mm) at which sporulation could be detected from the inoculum source, determined the growth of *T. harzianum*. In some experiments a visual assessment was made of the intensity of *T. harzianum* sporulation on a scale of sparse (+) to profuse (+++).

2.1.2 Quantification of T. harzianum growth in mushroom compost

T. harzianum spores were inoculated on rye grain and covered by mushroom compost, compressed by hand and incubated as described above. Growth of *T. harzianum* was determined by quantification of colony forming units (cfu) that were retrieved from a sample of compost. The compost was carefully removed from the tube and a depth of 1

cm containing the inoculum source was discarded. A sample of 10 g was taken from the remaining compost and added to 100 ml 0.1M sodium tetrapyrophosphate (Sigma, UK) in distilled water (Cazemier *et al.* 1997). Samples were steeped for 60 minutes at room temperature before 2 minutes continuous agitation in a Stomacher Lab-Blender 400. Finally, a 1 ml aliquot was removed and diluted 1:9 ml in 0.1M sodium tetrapyrophosphate. The resulting suspension was then serially diluted to give a log dilution series.

The detection of *T. harzianum* cfu required a selective medium that also reduced the growth of *T. harzianum* to give discrete colonies (see Chapter Five). The final medium, *Trichoderma* selective medium (TSM), was adapted from Askew and Laing (1993) is described below.

TSM:

Basal medium (l^{-1})

$MgSO_4.(7H_2O)$	0.2g
K_2HPO_4	0.9g
NH_4NO_3	1.0g
KCl	0.15g
rose bengal	0.15g
glucose	3.0g
agar	20.0g

in 950 ml distilled water; autoclaved at 121°C for 15 minutes

Antimicrobial and fungicidal ingredients (l^{-1})

chloramphenicol	0.25g
streptomycin stock (1% w/v in SDW)	9.0 ml
quintozene	0.2g
propamocarb	1.2 ml

in 39.8 ml sterile distilled water and added to cooled basal medium

(all chemicals Sigma, UK; except propamocarb, kindly supplied by Levington, UK)

TSM was inoculated with 100 µl of cfu dilutions and incubated at 25°C for 4 days. Number of colonies was determined using a Stuart Scientific colony counter.

2.2 Enzyme methodologies

2.2.1 Production of T. harzianum depolymerases in liquid cultures

Cultures of *T.harzianum* were grown in a basal medium (C+W) described by Cooper and Wood (1975) buffered to pH 6 with 2-(N-morpholino) ethanesulphonic acid (MES) and supplemented with 1% glucose (w/v). Three replicate 250 ml conical flasks containing 100 ml of medium, were inoculated with 1 ml of a 1 x 10^7 spores ml^{-1} suspension. These were incubated at 25°C with shaking at 180 rpm in the absence of light. After incubation for 3 days, the extensive mycelium (*ca.* 500 mg) was washed in 50 ml of sterile, unsupplemented C+W basal medium and transferred into 100 ml C+W basal medium (Cooper and Wood 1975). Cultures were incubated at 25°C overnight with shaking to starve the mycelium of glucose. Finally the mycelium was transferred into the 100 ml of medium containing a suitable inducer (1% w/v): *A. bisporus* cell wall extract to induce chitinase, laminarinase and protease and chopped wheat straw to induce cellulase, xylanase, laminarinase and protease activities. Flasks were returned to the 25°C shaking incubator and samples of culture fluids were removed after 12, 24, 48 and 72 hours and clarified by centrifugation at 3000x g. The mycelial growth was determined as dry weight.

Basal medium (Cooper and Wood 1975)

$NaNO_3$	0.2 % w/v
KH_2PO_4	0.1 % w/v
$MgSO_4.7H_2O$	0.05 % w/v

Trace element solution (100x stock)

$FeSO_4.7H_2O$	0.2 ppm
$ZnSO_4.7H_2O$	1.0 ppm
$NaMoO_4.2H_2O$	0.02 ppm
$CuSO_4.5H_2O$	0.02 ppm
$MnCl_2.4H_2O$	0.02 ppm

9.76g/l MES (2-(N-morpholino) ethanesulphonic acid) 50mM pH to 5.5 with NaOH

The production of chitinase, laminarinase and protease was achieved using mushroom cell walls (CW) as an inducer supplement. The CW preparation was a modification of a method described by Wang and Bartnicki-Garcia (1970). Button mushrooms were blended in cold 50mM Tris-HCl buffer at pH7 in the ratio 1 volume fungus (fresh weight) to 3 volumes buffer. The suspension was then centrifuged at 4000 xg for 6 minutes and the pellet resuspended in buffer. The centrifugation and washing was repeated 3 times followed by resuspension twice in distilled water. The crude cell walls were then lyophilised, sterilised by autoclaving and stored at -20°C. Induction of xylanase, cellulase and protease, was achieved by supplementing C+W medium with wheat straw blended in a coffee bean grinder. Wheat straw was used, in its crude form, which may simulate the form used in the composting procedure. The inducers were added at 1% (w/v) to the basal medium (C+W).

2.2.2 Production of T. harzianum depolymerases in sterile mushroom compost

Compost was autoclaved at 121°C for 1 hour and then left at room temperature overnight to allow germination of heat activated thermophiles. The autoclave procedure was repeated twice. Tubes were inoculated with *T. harzianum* conidia on sterile rye grain and filled with *ca.* 30 g sterile compost, compressed by hand. Lids were released a quarter turn to prevent the development of anaerobic conditions and incubated at 25°C, with high relative humidity and in the absence of light. Samples were collected after 1, 2 and 3 weeks. Enzymes were retrieved from compost samples in an extraction buffer designed to prevent denaturation of the enzymes and to allow maximum desorption from the compost substrate (Cooper and Wood 1980). The extraction buffer consisted of 5 mM dithiothreitol (to prevent oxidation), 0.2 M KCl (allowing desorption of proteins from cell walls) and 5% polyvinyl pyrrolidone (to adsorb phenols) in 50 mM sodium phosphate [pH 6.0] (all chemicals, Sigma, UK).

Cold extraction buffer was added to compost in the ratio 5ml : 1g (v/w fresh wt.). Samples were agitated for 1 minute in a Stomacher 'Lab-Blender 400', steeped on ice for 15 minutes and finally agitated for a further minute in the blender. Large solid matter was removed by filtration through 2 layers of muslin, subsequently the filtrate was clarified by centrifugation at 4,000g for 15 minutes. The supernatant was then subjected to centrifugation at 23,000g for 15 minutes. The resultant supernatant was

dialysed exhaustively against 25mM MES pH6 at 4°C, overnight. Finally, extracts were concentrated *ca.* 5-fold against 30,000 M_r PEG (Fluka,UK) at 4°C.

Before isoelectric focussing on gels, the samples were passed through PD-10 columns (Pharmacia Biotech) pre-packed with G-25 Sephadex for further desalting. The columns were calibrated using a blue dextran solution to determine the volume of liquid required to elute the sample. The sample (2 ml) was added to the column and once it had passed into the column, 0.8 ml of buffer (50 mM MES pH 6.0) was added to saturate the column. Finally, 2.7 ml of buffer was added to elute the sample.

2.3 Protein separation and purification

2.3.1 Ammonium sulphate precipitation

Combined culture fluids from three replicate flasks and the enzymes extracted from sterile compost were separately subjected to ammonium sulphate precipitation to concentrate the protein content. Solid ammonium sulphate was gradually added to 73% (w/v) saturation, to culture fluids stirred at 4°C. Centrifugation at 14, 000g for one hour at 4°C produced a pellet of precipitated protein. The pellet was resuspended in 50ml 25 mM MES [pH 6.0]. Samples were dialysed against 25 mM MES [pH 6.0] at 4°C overnight to remove ammonium sulphate.

2.3.2 Preparative Rotofor™ IEF

The electrodes were prepared by the addition of 25ml of 0.1M sodium hydroxide and 25ml of 0.1M phosphoric acid respectively, to the cathode and anode. Ampholytes [2%, pH 3-10] (BioRad) were added in a volume of 500µl to the dialysed protein sample (described above 2.3.1) and the volume made up to 55ml with 25 mM MES [pH 6.0], which was then injected into the focussing chamber. The Rotofor was run at 12W constant power at 4°C. Voltage was recorded every 30 minutes until it stabilised (typically 3 hours), at which point 20 fractions were collected *via* a collection chamber attached to a vacuum pump. The pH of each fraction was recorded and enzyme activity and protein content assayed.

2.3.3 Gel electrophoresis IEF

Dialysed samples (described above 2.3.1) were assayed for total protein content by the BioRad assay (see below 2.3.7). Volumes containing 100μg protein were then flash-frozen in liquid nitrogen and lyophilised, prior to storage at -20°C. Proteins were separated according to their isoelectric charge on 5% Ampholine PAG plate gels (Pharmacia Biotech). Broad range pH gels were used; pH 3.5-9.5 on a LKB Bromma 2117 Multiphor flatbed gel tank. Samples were focused at 4°C for 1.5 hours at 30 volts (15 volts for half gel) according to manufacturer's instructions. Sodium hydroxide (1M) was applied to cathode strip and 1M orthophosphoric acid was applied to anode. The anode and cathode strips were cut to the exact size of the gel and laid in position. Application pieces were placed on the gel at position 4 to which samples, resuspended in 10 μl milliQ water, were added. The application pieces were removed after half the total focusing time. Focused samples could then be stained to reveal the protein bands or overlaid with an activity gel to reveal the substrate specificity of the isoforms separated.

2.3.4 Detection of proteins on IEF gel

Once focussed, the electrode strips were removed carefully with forceps. Staining with Coomassie blue enabled the visualisation of the proteins. The gel was submerged in the following solutions: i) fixing solution, 30-60 minutes; ii) washing solution, 5 minutes; iii) staining solution, 2-3 hours; iv) destaining solution until background was clear (see Appendix A.5).

2.3.5 Determination of iso-electric points

A sample of proteins of known iso-electric points (pI) was also separated on the IEF gels to enable calibration. Once stained, the individual bands were recorded as a measurement of the distance migrated from the cathode. A calibration curve was then plotted of known pI against distance migrated from cathode and migration distances of samples could be converted to pI.

2.3.6 Detection of specific enzyme activities following IEF gel electrophoresis

The IEF gel was overlaid with a gel of 1% (w/v) agarose in 50 mM MES [pH 6.0] containing a dye-complexed substrate to determine the specific enzyme activity of the separated proteins. Gels were cast in polyacrylamide gel plates with 1 mm spacers.

Once cut to size, gels were applied directly to the IEF gels when care was taken to remove all air bubbles and close contact between gels was ensured.

Remazol Brilliant Blue (RBB)-curdlan, RBB-gelatin and carboxymethyl-chitin-Remazol Brilliant Violet (Loewe GmbH, Germany) were used to detect laminarinase, protease and chitinase activities, respectively. The substrates (4 ml of a 4 mg ml^{-1} stock) were incubated in a 75°C water bath and subsequently mixed with 1 ml of agarose solution also at 75°C (5% w/v agarose in 50 mM MES pH 5.5). The overlaid IEF gels were then incubated at 37°C for 30 minutes. Agarose overlays were removed and destained (1:2 v/v, 50mM acetate buffer (pH5.4):ethanol (96%)).

Cellulases were detected using Ostazin Brilliant Red-hydroxyethylcellulose (OBR-HEC) (Sigma, UK) and 4-methylumbelliferyl-β-D-cellobioside (MUC) (Sigma, UK). The substrates (0.05% w/v OBR-HEC or MUC) were dissolved in a solution of molten 1% (w/v) agarose in 50 mM MES [pH 5.5]. Overlaid IEF gels were incubated at 50°C for 30 minutes or 10 minute intervals for OBR-HEC and MUC respectively. OBR-HEC gels were removed and destained (1:2:1 (v/v), 50mM acetate buffer (pH5.4):ethanol (96%)-acetone) (Biely *et al.* 1985). Gels containing MUC were removed at 10 minute intervals and placed under a UV light source to check for fluorescence; typically periods of 20 minutes were adequate. To distinguish β-glucosidase activity from the other cellulases (Chapter Five 5.1.3(iii)) two replicate samples were focussed, one of which was soaked in 10 mM gluconolactone (Sigma, UK) in 50 mM MES [pH5.5] for 10 minutes prior to addition of activity gel containing MUC. Gluconolactone inhibited β-glucosidase activity.

RBB-xylan (Sigma, UK) was used to detect xylanase activity. The substrate (0.05% w/v) was dissolved in a solution of molten 1% (w/v) agarose in 50mM MES [pH 5.5]. After 30 minutes incubation at 50°C, the activity gels were removed and destained [1:2 (v/v), 50mM acetate buffer (pH 5.4):ethanol (96%)] (Biely *et al.* 1985).

Finally, overlay gels with achromatic zones to indicate enzyme degradation were preserved by the addition of 5% trichloroacetic acid to the destain solution to denature the enzymes. Gels were then sealed in plastic with glycerol and stored at 4°C.

2.3.7 Total protein quantification

Protein concentration of samples concentrated by ammonium sulphate precipitation and PEG was estimated using the BioRad assay. BioRad reagent (50 µl) was added to 200 µl of sample on a microtitre plate and mixed well. The plate was centrifuged to remove air bubbles and then incubated at room temperature for 15 minutes. The $A_{595\ nm}$ was determined against 50 mM MES [pH 6.0] blanks using a Dynatech MR5000 plate reader. The absorbance readings were then converted into concentrations (µg protein) using a calibration curve of readings taken from known concentrations of bovine serum albumin (BioRad).

2.4 Enzyme Assays

2.4.1 Laminarinase, chitinase, cellulase and xylanase

Glucanase, chitinase, cellulase and xylanase were assayed by following the release of reducing sugars from the relevant substrate (Nelson 1944; Somogyi 1952). Clean, acid-washed glassware was used for all stages.

The substrates, 1 mg/ml laminarin (Sigma, UK), birch wood xylan (Sigma, UK) and 25 mg/ml colloidal chitin (see Appendix A.3), were made up in 50mM MES pH5.5. Cellulose, (1 mg/ml) in the form of insoluble filter paper (Whatman No.1), was blended in 50mM MES pH6.0. The substrates and enzyme samples were separately pre-incubated at the assay temperatures (see below). After 5 minutes, 500µl of the substrate was added to 100 µl enzyme samples and the test tubes were incubated at 37°C (laminarinase and chitinase activities) or 50°C (cellulase and xylanase activities). All reactions were incubated for periods previously determined as optimal for detecting enzyme activity; i.e. laminarinase, cellulase and xylanase for 20 minutes and chitinase for 60 minutes. Reactions, including the blanks, were terminated with 500µl of stopping reagent (see Appendix A.4); caps were placed on the tubes and incubated at 95°C for 30 minutes to develop the copper complex. Once the tubes had cooled, 500µl of Nelson C reagent (see Appendix A.4) was added and the tubes vortexed and left for 2 hours for the colour to develop.

Time zero controls were produced by immediately adding 500µl of stopping reagent to 100µl of the test sample; subsequently, samples were treated the same with the addition

of substrate and Nelson C. Enzyme blanks consisted of 100µl of 50mM citrate buffer pH6 in place of the test sample.

The absorbance at 595nm, against citrate buffer blanks, was then determined using a Dynatech MR5000 plate reader. In order to convert the value of A_{595nm} into activity units (nkat), a standard curve for the predominant monosaccharide from each substrate was produced. Glucose, xylose and *N*-acetylglucosamine, the monomers of laminarin and cellulose, xylan and chitin respectively, were used at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 mM. Calibration curves of sugar concentration (mM) against A_{595nm} were produced (see Appendix A.7) and sample absorbencies were converted to concentrations. The concentrations were then converted into units since 1 unit was defined as the amount of enzyme required to convert 1 µmole per minute. Finally, this value was used to calculate the number of Katals since 1 unit of enzyme is equivalent to 16.67 nKats.

2.4.2 Azocasein Protease Assay

This is based on the release of a red dye from a protein substrate. The azocasein (Sigma, UK) substrate was dissolved in 50mM MES pH 5.5 to produce a 3% (w/v) solution. Both substrate and test samples were pre-incubated at 37°C. To 50 µl test samples, 100 µl of azocasein substrate was added and the reactions incubated for a further 180 minutes at 37°C. Controls included an enzyme blank in which 50µl of buffer was added instead of test sample and a boiled, inactivated enzyme test sample. The reaction was stopped with 500µl of 20% trichloroacetic acid (TCA), then mixtures were vortexed, centrifuged at 13 000g for 2 minutes and a 200µl sample was removed and the absorbance measured against the buffer blanks at 405nm by a Dynatech MR5000 plate reader.

2.4.3 Pr1 and Pr2 Protease Assay

The proteases Pr1 (chymoelastase) and Pr2 (trypsin-like protease) previously characterized from *Metarhizium anisopliae* (St Leger *et al.* 1987a) degrade the substrates Suc-$(Ala)_2$-Pro-Phe-pNA (Sigma, UK) and Bzoyl-Phe-Val-Arg-pNA (Sigma, UK) respectively. On degradation, the yellow dye released can be detected at 405nm. These substrates were used to investigate possible similar protease activities by *T.harzianum* under inducing conditions. The assays were performed in microtitre plates and the substrates suspended in dimethyl sulphoxide (DMSO). The test volumes

comprised: 40µl test sample, 160µl Tris-HCl buffered to pH 8.0 (0.225M) and 50µl substrate. Substrates were added using a multi-channel pipetter. Absorbency readings were taken on a Dynatech MR5000 plate reader once every 30 seconds for 3 minutes or after 1, 3, 5, 7 and 10 minutes, depending on the activity of the sample. Controls replacing samples with Tris-HCl buffer were included. The test samples often appeared yellow-brown prior to the assay and therefore reactions replacing substrate with buffer were used to record background absorption. A calibration curve was produced using *p*-nitroaniline dissolved in DMSO in the following concentrations: 15.63, 31.25, 62.5 and 125 µM. The reaction consisted of 90µl standard and 160µl Tris-HCl buffer. Absorption was recorded at 405nm. The concentration of *p*-nitroaniline was plotted against absorbance (see Appendix A.7), then sample absorbance readings were converted into concentrations (µM).

2.4.4 Effect of inhibitors on Prl- and Pr2-like activities

Samples of culture filtrates (20 µl) from *T. harzianum* strain T7 induced by *A. bisporus* cell wall extract for 24 hours, were incubated with various protease inhibitors (20µl) at room temperature for 30 minutes. Commercially available inhibitors (Sigma, UK) were chosen to cover five classes of protease: ethylenediaminetetraacetic acid (EDTA) (50mM, water pH8.5) inhibits metallo-proteases by chelating active site zinc ions; Iodoacetic acid (IAA) (50 µM in water) irreversibly inhibits cysteine proteases; leupeptin (50 µM in water) inhibits trypsin-like serine proteases and some cysteine; pepstatin (1 µM in DMSO) inhibits aspartic proteases and phenylmethylsulphonyl fluoride (PMSF) (1 mM in methanol) irreversibly inhibits serine proteases (Beynon and Salvesen 1989). The solvents in which the inhibitors were dissolved were included as controls and incubated with samples for 30 minutes at room temperature. After incubation the assay was performed as described above (section 2.4.3) and A_{405nm} was determined after 1, 3, 5, 7 and 10 minutes with a Dynatech MR5000 plate reader.

2.5 Molecular methodologies

2.5.1 Preparation of genomic DNA

Mycelial plugs (3 mm diameter) of *T. harzianum* and *A. bisporus* were inoculated on cellophane disks previously overlaid onto MEA. Plates were incubated at 25°C in the absence of light until the cellophane disks were covered with mycelium, typically 3

days or 3 weeks for *T. harzianum* and *A. bisporus* respectively. Mycelia were removed, flash-frozen in at -70°C and then ground in liquid nitrogen using a pre-chilled (-70°C) pestle and mortar. Genomic DNA was extracted using a modification of the procedure described by Raeda and Broda (1985). Ground mycelium from five petri dishes was dispensed into an oakridge centrifuge tube to which 10 ml of fungal extraction buffer (FEB; 200 mM Tris-HCl [pH 8.5], 250 mM NaCl; 25 mM EDTA, 0.5% w/v SDS) was added and gently mixed by shaking. 7 ml phenol was added and the tube gently mixed, followed by 3 ml chloroform-isoamyl alcohol (24:1 v/v) equilibrated with FEB and the mixture was gently shaken. The mixture was centrifuged at 11000g for 30 minutes in the temperature range 10 to 30°C. The aqueous phase was decanted into a clean oakridge tube and the phenol extraction step repeated. The aqueous phase was again transferred to a fresh tube containing 500 µl of DNase-free RNase and incubated at 37°C for 15 minutes. Subsequently, 10 ml chloroform/isoamyl alcohol was added and the tube contents were mixed by gentle shaking, then centrifuged at 11000g at room temperature for 15 minutes. The aqueous phase was transferred to a clean tube and 5 ml isopropanol added to precipitate the DNA, mixing was achieved by inversion. Centrifugation at 7000g at room temperature for 15 minutes pelleted the DNA. The pellet was washed with 70 % v/v ethanol and centrifuged briefly. Discarding the supernatant, the pellet was left to air dry. Pellet was rehydrated in 500 µl of TE buffer (10 mM Tris-Hcl [pH 8.0], 1 mM EDTA) and stored at 4°C.

2.5.2 Polymerase chain reaction (PCR) and gel electrophoresis

Primers, designed to *cell* gene of *A. bisporus* (Raguz *et al.* 1992), were as follows: Forward: 5', gAC gTC CCg gTg TgA CCA (designed using PrimerCalc™, Molecular Sensing Plc, UK) and Reverse: 5', TAC ATg AgC gAT CAT (previously designed, supplied by Dr P Mills, HRI Wellesbourne). Using the software package First Step™ (Molecular Sensing Plc, UK), the following optimal conditions were calculated: denaturation at 94°C for 30 seconds, 2 minutes at an annealing temperature of 50°C followed by extension of 30 seconds at 72°C. This sequence was repeated for 35 cycles.

The components of the PCR mixture were optimised using Taguchi beta site testing (Cobb and Clarkson 1993). This allowed several variables to be considered using just 9 reactions. Nine pre-prepared reaction mixes (Molecular Sensing Plc, UK) contained

nucleotides, $MgCl_2$, buffer and Taq polymerase (in 30µl). Primers, template and milliQ water were added in a volume of 20µl to give a final volume of 50µl. Concentrations of the components in the 9 reactions are described in Table 2.2.

PCR products were analysed by gel electrophoresis on a 1.5% w/v agarose gel in 0.5x TBE. Subsequently the products for each reaction were given a score between 1 and 10 (10 = highest fluorescence) and the signal-to-noise (*SNL*) ratio calculated using First Step™. Optimal component concentration was determined by the largest *SNL* and polynomial regression used for further accuracy (Cobb and Clarkson 1993). Optimal reaction concentrations were as follows: 5.0 µl, 10x reaction buffer; 5.0 µl, 2 mM dNTPs; 2.0 µl, 50 mM $MgCl_2$; 2.5 µl, 10 pmole primers; 2.0 units Taq; 1.0 µl, 10ng μl^{-1} template DNA and 30.0 µl milliQ water.

2.5.3 PCR product purification and sequencing

PCR products were purified from 1.5% agarose gels using a Sephaglas™ BandPrep Kit according to manufacturer's instructions (Amersham Pharmacia Biotech, UK). The PCR product was excised from the gel and weighed. Gel solubilizer (250 µl) and 5 µl acetic acid were added and the mixture was vortexed vigorously prior to incubation at 60°C for 10 minutes to dissolve the agarose. 5 µl of vortexed Sephaglas BP was added and the dissolved gel slice was incubated at room temperature for 5 minutes with gentle vortexing every minute to resuspend the Sephaglas. The mixture was centrifuged for 30 seconds and the supernatant was discarded. The Sephaglas pellet was washed in 80 µl of wash buffer, centrifuged for 30 seconds and the resulting supernatant removed. This washing step was repeated twice. The pellet was air-dried for 10 minutes prior to the addition of 20 µl of elution buffer. The pellet was dispersed by vortexing and left at room temperature for 5 minutes with periodic agitation. Finally, centrifugation at high speed for 1 min pelleted the Sephaglas and the supernatant containing the DNA was removed and stored at 4°C.

Reactions (6 µl final volume) containing 30 ng of DNA and 3.2 pmole of a primer were set up to sequence the amplification products. PCR products from genomic DNA of *A. bisporus* strain A12 and *T. harzianum* strains Th1(c), T7 and RM10c were sequenced on ABI Prism Model 377 Version 2.1.1 (Jones P, Sequencing Unit, University of Bath).

Table 2.2. Components of the Taguchi Beta Site Testing 9 Reactions

Reaction	dNTPs* (mM)	$MgCl_2$* (mM)	Taq* (units)	Primers* (pMol)	Primers[a] (µl)	DNA[†a] (µl)	milliQ[a] (µl)
1	0.1	1.0	0.5	10	1+1	1.0	17
2	0.1	2.0	1.25	20	2+2	1.0	15
3	0.1	3.0	2.0	30	3+3	1.0	13
4	0.2	1.0	1.25	30	3+3	1.0	13
5	0.2	2.0	2.0	10	1+1	1.0	17
6	0.2	3.0	0.5	20	2+2	1.0	15
7	0.3	1.0	2.0	20	2+2	1.0	15
8	0.3	2.0	0.5	30	3+3	1.0	13
9	0.3	3.0	1.25	10	1+1	1.0	17

* Final concentration of component in reaction.
[a] Volume added to reaction to give desired final concentration.
[†]10 ng template added to each reaction in a volume of 1 µl.

2.6 Electron microscopy

2.6.1 Cryo-scanning electron microscopy

Scanning electron microscopy (SEM) provided an image with increased depth of field and was capable of much higher magnification compared to the light microscope. Samples were removed, attached to the SEM stub and then flash frozen in liquid nitrogen. The sample stub was introduced to the initial chamber where any ice was removed by subliming the liquid nitrogen. Subsequently, the sample was sputter coated with gold and introduced to the main chamber.

2.6.2 Energy disperse system (EDS)

The chemical composition of the crystals observed on the mycelial surface of *A. bisporus* was determined using EDS by x-ray microanalysis of a specific region of the mycelium. The sample was positioned at a fixed distance from the x-ray detector and a beam of primary electrons was focussed onto the crystals. The x-rays produced when the electrons hit the mycelial surface were detected and analysed by computer. Chemical composition was determined by the energy level of the x-ray wavelengths.

Chapter Three

Investigations into possible antagonism involved in interactions between *Trichoderma harzianum* and *Agaricus bisporus*.

3.0 Introduction

The growth of *Agaricus bisporus* is reduced by the presence of aggressive genotypes of *Trichoderma harzianum* in mushroom compost (Seaby 1987). *T. harzianum* colonises compost and manifests as large patches of green mould, which are devoid of mushrooms. Such infections or infestations often result in failure of the complete crop. While the literature has suggested the possibility of an antagonistic relationship (Seaby 1987), there has been no demonstration of a particular mechanism. The complexity of the compost environment and the ability of the potential antagonist, *T. harzianum* to adopt changing strategies in order to survive in highly competitive habitats, suggest that the mode(s) of antagonism may be difficult to elucidate.

Compost is selective for *A. bisporus* and therefore to have a negative affect on its growth, aggressive *T. harzianum* genotypes must have either adapted to such an environment or evolved mechanisms to overcome the selective nature of this substrate. One such method would be to increase its competitive saprophytic ability. This could be achieved by more efficient colonisation of the compost, perhaps by utilising cellulases and xylanases to obtain nutrients from the straw component of compost. Also *T. harzianum* may switch from saprophytic mode to mycoparasitism and obtain nutrients directly from and at the expense of *A. bisporus*.

An extensive microflora exists within mushroom compost and therefore the potential number of interactions is high, however not all will have an aggressive or combative result. Species of *Trichoderma* produce a wide range of antifungal compounds and are particularly successful in soil environments. The production of volatile and non-volatile antibiotics by *Trichoderma* spp. has been described and has been suggested to have important roles in antibiosis and are investigated in this section (Dennis and Webster 1971a, 1971b). A wide array of depolymerising enzymes are produced by *T. harzianum*

that have activity against polymers present in cell walls of fungi (Elad *et al.* 1982, 1984; Ridout *et al.* 1988), and plants (Wood and Garcia-Campayo 1990). Early studies revealed the importance of hyphal interactions in the mycoparasitism of plant pathogens by *T. harzianum* (Dennis and Webster 1971c). Ultrastructural studies have revealed the production of parasitic structures (Elad et al 1983a; Benhamou and Chet 1993) and morphological features (Barak *et al.* 1985) involved in the penetration or non-invasive parasitism of hosts.

This chapter attempts to establish the nature of potential antagonistic mechanisms employed by *T. harzianum* in its negative effects towards the growth of *A. bisporus*. Attention has been paid to possible interactions at both colony and hyphal levels. To increase validity, both *in vitro* and *in vivo* models were designed. The dual model system allowed an initial rapid study of several genotypes and strains that could be assessed semi-quantitatively (*in vitro*). Subsequently, a scale model of the green mould problem attempted to mimic interactions in commercial compost and allowed consideration of other influences such as microflora and changing humidity that can not easily be replicated for *in vitro* agar plate assays. The scale model also considered various routes and sources of infection.

3.1 Results

3.1.1 The effect of the introduction of T. harzianum to cultures of A. bisporus on various solid media

The media chosen represented different environments to investigate any effect the available nutrients had on the interactions. The media (see appendix) used were, malt extract agar (MEA), distilled water agar (DWA), filtered compost agar (fCA) and blended compost agar (bCA). Submerged growth by *T. harzianum* was prevented using a cellophane membrane, previously sterilised and placed directly on the agar surface prior to inoculation. These media were used throughout the following *in vitro* assays.

The fungal isolates used throughout *in vitro* assays were A12, a white, susceptible strain of *A. bisporus* and *T. harzianum* isolates Th1(c) and TD15 (Th1) and T7, T7 reduced, Th2f (Th2).

Statistical analyses (P. Christy, pers. comm.) were performed using Minitab for Microsoft Windows. Diameters of control cultures (pure cultures) were compared to diameters of "treated" dual cultures on each medium at each particular time measured. This was achieved using unpaired, two-tailed t-tests ($P<0.05$) for parametric data and Mann-Whitney test for non-parametric data. Genotype comparisons were performed for each medium at each time point. Prior to analysis each genotype replicate was corrected by subtracting the "treated" diameters from "control" diameters. One-way analysis of variance (ANOVA) was used to compare parametric data of more than 2 strains followed by Bonferroni's multiple comparison test if the analysis was significantly different ($P<0.05$) (Prism® Graph Pad software). Non-parametric data was analysed using Friedman test (> 2 strains) followed by Dunn's multiple comparison post test.

3.1.1(i) Investigation of competition between T. harzianum and A. bisporus in dual cultures

Agar media were initially inoculated with a 3 mm cork borer plug of *A. bisporus* (A12) mycelium and subsequently incubated at 25°C for 10 days (MEA, DWA) or 8 days (fCA). The initial pure culture incubation period allowed the establishment of *A. bisporus* after which, the petri dish was inoculated with a 3mm diameter plug of *T. harzianum* mycelium at a distance of 3cm from the *A. bisporus* colony (Royse and Ries 1978). Dual inoculated plates were returned to the 25°C incubator and growth of the two fungal colonies was followed by measuring the diameter at two perpendicular

points. Control plates were inoculated with the individual strains of the fungi and for each treatment, five replicates were produced.

On DWA, *T. harzianum* appeared to have limited effects on *A. bisporus* growth. Initially (for 48 hours) *T. harzianum* strain T7 had no effect on the growth of *A. bisporus* however, after 3 days incubation growth was significantly inhibited by T7 (t-test, P = 0.0235; F-test not significant – ns). *T. harzianum* strain Th2F (Th2) appeared to stimulate *A. bisporus* growth after 2 days incubation (t-test, P = 0.0485; F-test, P = 0.0426) but failed to differ from controls at other times. The non-aggressive *T. harzianum* strains Th1(c) and TD15 (Th1) had no inhibitory or stimulatory effects on *A. bisporus* growth on DWA (Table 3.1).

Agaricus bisporus growth appeared, initially to be significantly inhibited by both genotypes (1 and 2) on MEA, however incubation beyond 24 hours revealed no difference between treatments and controls (Table 3.1). The presence of *T. harzianum* strain A006022 (Th3) stimulated the growth of *A. bisporus* growth on fCA after 24 hours incubation (t-test, P = 0.0365; F-test, P = 0.0059) (Table 3.1). After 48 hours incubation there was no discernible differences between any of the treatments and controls. A significant inhibitory effect was detected after 3 days incubation when *A. bisporus* was cultured with *T. harzianum* strain Th1(c) (t-test, P = 0.0085; F-test, ns), T7 (t-test, P = 0.0025; F-test, P = 0.0183) and Th2F (t-test, P = 0.021; F-test, ns).

Trichoderma harzianum also exhibited some growth changes in the presence of *A. bisporus*. On DWA, strain T7 (Th2) displayed significant inhibition after 24 (t-test, P = 0.0022; F-test, P = 0.0006) and 48 (t-test, P = 0.0158; F-test, ns) hours incubation with *A. bisporus* (Table 3.2) however, after 3 days incubation the effect was undetectable. *T. harzianum* strains TD15 (Th1) (t-test, P < 0.0001; F-test, P = 0.0352) and Th2F (Th2) (t-test, P = 0.0044; F-test, ns) were significantly inhibited by *A. bisporus* on DWA after 2 and 3 days incubation (Table 3.2). Interactions on MEA appeared to inhibit significantly Th1 at all times with the exception of strain TD15 after 24 hours incubation (Table 3.2).

Table 3.1. Colony growth of *A. bisporus* in pure cultures and dual cultures with *T. harzianum*.

Medium	Time (days)	Growth of *A. bisporus* (mean diameter (mm) ± SEM)				
Isolate pairings		A12	A12 α Th1(c)	A12 α TD15/ A006022	A12 α T7	A12 α Th2F
DWA	1	21 ± 1	21 ± 1	22 ± 0	19 ± 1	22 ± 1
	2	23 ± 1	23± 1	24± 1	22 ± 1	25 ± 0†
	3	24 ± 1	23± 1	24± 1	21± 1*	26 ± 0
MEA	1	27 ± 2	22 ± 1*	24± 2	21 ± 0*	24 ± 3
	2	26 ± 2	24± 1	23± 0	23 ± 1	23± 1
	3	27 ± 2	24± 1	25 ± 0	23± 1	25 ± 0
fCA	1	20 ± 4	26± 1	30 ± 1††	26± 1	26± 2
	2	33 ± 2	29± 1	32± 1	33 ± 2	30 ± 1
	3	38 ± 1	29 ± 2*	34± 2	33 ± 0**	33 ± 1*

† indicates *A. bisporus* growth was significantly higher than the control, according to unpaired, two-tailed t-tests ($P<0.05$). †† indicates that *A. bisporus* growth was significantly higher than control according to t-test and F-test (<0.05).

* indicates *A. bisporus* growth was significantly lower than the control, according to unpaired, two-tailed t-tests ($P<0.05$). ** indicates that *A. bisporus* growth was significantly lower than control according to t-test and F-test (<0.05).

NB. *T. harzianum* strain A006022 (genotype 3) was used on fCA in place of strain TD15 (Th1).

Table 3.2. Colony growth of *T. harzianum* in pure cultures and in dual cultures with *A. bisporus*.

Medium	Time (days)	Growth of *T. harzianum* (mean diameter (mm) ± SEM)							
Isolate pairings		Th1(c)	Th1(c) α A12	TD15/ A006022	TD15/A006022 α A12	T7	T7 α A12	Th2F	Th2F α A12
DWA	1	20± 0	20 ± 0	13± 0	12± 1	21 ± 0	17 ± 1**	15± 1	13 ± 1
	2	58 ± 1	54 ± 2	60 ± 1	55± 0**	59± 1	56± 0*	49± 1	42 ± 2*
	3	70± 1	61± 1*	73± 1	66 ± 1*	70± 2	65± 2	60± 1	52 ± 2**
MEA	1	26± 0	23± 1*	19 ± 1	18 ± 1	26 ± 1	25± 0	17± 0	21± 3
	2	72 ± 1	64 ± 1*	68± 1	58 ± 2*	69 ± 0	61 ± 1*	60± 1	56 ± 2
	3	79± 1	73 ± 2**	78± 0	69± 2**	78 ± 1	74± 1*	73± 1	63 ± 1*
fCA	1	19± 0	19± 0	11± 0	10 ± 1	17 ± 0	17± 2	14± 1	15 ± 3
	2	47 ± 1	47± 1	30± 1	27± 3	44± 1	42 ± 4	38 ± 1	34± 2
	3	68 ± 0	61 ± 0*	52± 2	47± 4	67 ± 2	57± 3*	59± 1	53 ± 3

† Indicates *T. harzianum* growth was significantly higher than the control, according to unpaired, two-tailed t-tests ($P<0.05$). †† Indicates that *T. harzianum* growth was significantly higher than control according to t-test and F-test (<0.05).

* Indicates *T. harzianum* growth was significantly lower than the control, according to unpaired, two-tailed t-tests ($P<0.05$). ** Indicates that *T. harzianum* growth was significantly lower than control according to t-test and F-test (<0.05).

NB. *T. harzianum* strain A006022 (genotype 3) was used on fCA in place of genotype 1 strain Th1(c). *T. harzianum* strains genotype 1: Th1(c), TD15; genotype 3: A006022; genotype 2: T7 and Th2F.

T. harzianum strain T7 was not inhibited initially on MEA, but exhibited significantly reduced growth in the presence of *A. bisporus* after 2 days (t-test, $P < 0.0001$; F-test, ns) incubation onwards (t-test, $P= 0.0211$; F-test, ns) (Table 3.2). Another Th2 strain (Th2F) was only significantly inhibited after 3 days incubation with *A. bisporus* (t-test, $P = 0.0035$; F-test, ns).

Dual cultures on fCA exhibited varied growth characteristics. Th2 appeared not to be affected by the presence of *A. bisporus* (Table 3.2). No significant differences were detected between treatments and controls for any *T. harzianum* strains up to 2 days incubation (Table 3.2). The presence of *A. bisporus* appeared to reduce the growth of *T. harzianum* strains A006022 (Th3) and Th2F (both Th2) after 3 days incubation (Table 3.2). A substantial inhibitory effect was detected towards *T. harzianum* strains Th1(c) (t-test, $P < 0.0001$; F-test, ns) and T7 (t-test, $P = 0.045$; F-test, ns) after 3 days incubation on fCA in the presence of *A. bisporus*.

Agaricus bisporus diameters from dual cultures were corrected against the *A. bisporus* control (i.e. dual culture diameters subtracted from controls) and the differences were used in one-way analysis of variance for each medium at each data collection time (see Appendix). This analysis revealed no significant difference between the effects of *T. harzianum* strains. Identical analysis was performed on differences between *T. harzianum* control and dual culture colony diameters and again this revealed no significant difference between *T. harzianum* strains with the exception of interactions on MEA after 24 hours (see Appendix). Under these conditions strains of *T. harzianum* exhibited significant differences ($P = 0.0082$) and Bonferroni's multiple comparison test revealed that strain Th1(c) appeared to be significantly reduced in growth compared to strain Th2F on MEA in the presence of *A. bisporus* ($P<0.05$).

In summary, *T. harzianum* appeared to have limited affects on the growth of *A. bisporus*. Th2 inhibited *A. bisporus* on all media with the exception of strain Th2F, which had a stimulatory effect on DWA and after 24 hours on fCA. Th1 also inhibited the growth of *A. bisporus*, however strain TD15 exhibited stimulatory effects towards *A. bisporus* on DWA and after 24 hours on fCA. The presence of *A. bisporus* produced differences in *T. harzianum* growth. Genotypes 1 and 2 were inhibited by *A. bisporus* to some extent on all media. When cultured on fCA, only limited effects were detected on

T. harzianum growth (genotypes 1, 2 and 3) and these were generally not significantly different.

3.1.1(ii) Production of non-volatile substances by T. harzianum

A mycelial plug (3mm diameter) of *T. harzianum* was inoculated centrally on an autoclaved, cellophane disc on each of the nutrient agar media (Dennis and Webster 1971). The inoculated plates were incubated at 25°C for 24 to 48 hours or until mycelial growth had covered the cellophane disc. The colonised cellophane disc was removed and the agar surface was centrally inoculated with a mycelial plug (3mm diameter) of *A. bisporus* and the plate returned to the 25°C incubator. Expansion of the colony was followed by recording of the diameter of the colony at two perpendicular sites. Controls were produced of *A. bisporus* on agar that had not been subjected to previous colonisation by *T. harzianum* on cellophane. Five replicates were produced for each treatment.

The growth of *A. bisporus* on agar media that had previously been inoculated with *T. harzianum* on cellophane discs was affected to varying extents dependent on the type of agar used (Table 3.3). After 6 days incubation on DWA, only *T. harzianum* strain T7 (Th2) had a significant inhibitory effect on *A. bisporus* growth (t-test, P = 0.003; F-test, ns). Strain T7 maintained the inhibitory effect after 7 days incubation (t-test, P = 0.0346; F-test, P = 0.011), while *A. bisporus* on DWA subjected to *T. harzianum* strain Th1(c) (Th1) exhibited significantly higher growth than the control (t-test, P = 0.0006; F-test, 0.0432).

On MEA, *A. bisporus* was significantly inhibited by all strains of *T. harzianum* at all data collection times. *T. harzianum* strain T7 exhibited a inhibitory effect on *A. bisporus* on fCA at all times (t-tests, $P < 0.05$; F-tests, ns) (Table 3.3). *A. bisporus* growth was unaffected by other strains of *T. harzianum* after 5 and 6 days incubation. However, after 7 days incubation *T. harzianum* strains Th1(c) (t-test, P = 0.0017; F-test, ns) and Th2F (t-test, P = 0.001; F-test, ns) both had inhibitory effects on the growth of *A. bisporus* (Table 3.3).

Table 3.3. Growth of *A. bisporus* in pure cultures and on agar previously inoculated with *T. harzianum* to investigate the possible production of non-volatile antibiotic compounds.

Medium	Time (days)	Growth of *A. bisporus* (mean diameter (mm) ± SEM)				
Isolate pairings		A12	A12 α Th1(c)	A12 α TD15	A12 α T7	A12 α Th2F
DWA	5	9 ± 0	9± 0		7± 1	8± 0
	6	11 ± 0	11± 0		9 ± 0*	10± 0
	7	13 ± 0	14 ± 0††		12 ± 1**	12± 0
MEA	5	13 ± 0	11 ± 0*	9 ± 0*	10 ± 0*	8± 0*
	6	15 ± 0	12± 0*	10± 0*	12 ± 0*	10± 0*
	7	16 ± 0	14± 0*	12 ± 0*	14± 0**	12± 1*
fCA	5	14 ± 0	13± 0	15± 1	12 ± 1*	12± 1
	6	19± 0	18± 0		15 ± 1*	16 ± 2
	7	23± 0	20 ± 0*		19 ± 1*	17± 1*

† Indicates *A. bisporus* growth was significantly higher than the control, according to unpaired, two-tailed t-tests (P<0.05). †† Indicates that *A. bisporus* growth was significantly higher than control according to t-test and F-test (<0.05).

* Indicates *A. bisporus* growth was significantly lower than the control, according to unpaired, two-tailed t-tests (P<0.05). ** Indicates that *A. bisporus* growth was significantly lower than control according to t-test and F-test (<0.05).

The effects of *T. harzianum* strains on the growth of *A. bisporus* were compared by performing one-way ANOVA on the differences between control diameters and those colonies potentially exposed to *T. harzianum* non-volatile antibiotic compounds (see Appendix). *T. harzianum* strain T7 had a significant inhibitory effect towards *A. bisporus* on DWA, in contrast strain Th1(c) had a stimulatory effect; when the differences were compared, the effects of the two strains were significantly different ($P<0.01$).

On MEA all the strains of *T. harzianum* used had significant inhibitory effects towards the growth of *A. bisporus* (Table 3.4). Also one-way ANOVA revealed significant differences between the strains of *T. harzianum* and their effects on *A. bisporus* (Table 3.4). *T. harzianum* strain Th1(c) (Th1) appeared to be significantly less inhibitory than strains TD15 ($P<0.05$) and Th2F ($P<0.01$) at all times. *T. harzianum* strain T7 (Th2) was significantly less inhibitory than Th2F at time periods 5 ($P<0.05$) and 6 days ($P<0.05$) and strain TD15 after 6 ($P<0.05$) and 7 days ($P<0/05$) incubation.

Agaricus bisporus growth on fCA was consistently inhibited by Th2 at all times however, only strain T7 exhibited significant inhibition. Comparison of the effects of all strains of *T. harzianum* at each time point revealed significant differences (Table 3.4). *T. harzianum* genotype T7 apparently differed significantly to strain TD15 which had slightly stimulated the growth of *A. bisporus* (Table 3.3).

To summarise, aggressive Th2 inhibited the growth of *A. bisporus* on all media with the exception of strain Th2F on DWA. Strain Th1(c) had inhibitory effects on *A. bisporus* on all media except DWA.

3.1.1(iii) Production of volatile substances by T. harzianum and A. bisporus

Media were inoculated with mycelial plugs of *A. bisporus*, placed centrally on the plates and incubated at 25°C. Once the *A. bisporus* colony had established (*ca.* 10mm diameter) the petri dish lid was removed and another plate, freshly inoculated with *T. harzianum*, was placed immediately opposite and the plates were sealed together using parafilm (Whipps 1987). Growth of *A. bisporus* was followed by measuring the colony diameters in two, perpendicular planes. Control plates were inoculated with individual

Table 3.4. One-way analysis of variance* to compare the *T. harzianum* strains and their effects on the growth of *A. bisporus*.

Medium	Incubation time (days)	P value (one-way ANOVA)
DWA	5	0.0042
	6	0.0102
	7	0.004
MEA	5	0.0025
	6	0.0025
	7	0.004
fCA	5	0.0259

*Significant results only – see Appendix (section B.3.1) for full analysis.

fungi and the plate was sealed opposite an uninoculated plate; five replicates were produced for each treatment.

The presence of *T. harzianum* strain T7 (fungi were separated spatially) had no effect on the growth of *A. bisporus* on DWA (Table 3.5). However, a non-aggressive strain of *T. harzianum*, Th1(c) appeared to inhibit the growth of *A. bisporus* after longer periods of incubation; typically after 3 days (t-test, P = 0.0353; F-test, ns) and 4 days (t-test, P = 0.0034; F-test, ns). On MEA, strain T7 (aggressive genotype) appeared to stimulate significantly *A. bisporus* growth at all data collection times. *T. harzianum* strain Th1(c) only exhibited stimulatory behaviour towards *A. bisporus* growth after 2 days incubation on MEA (t-test, P = 0.0135; F-test, ns) and had no effect on *A. bisporus* growth at other data collection times.

Incubation for 24 hours in the presence of *T. harzianum* strains on fCA had no detectable effect on the colony growth of *A. bisporus*. Conversely, *A. bisporus* appeared to be stimulated by the presence of *T. harzianum* strains at all times; however, significant stimulation was only exhibited by strain Th1(c) after 2 days (t-test, P = 0.0411, F-test, ns) and 4 days (t-test, P = 0.0133; F-test, ns) incubation and by strain T7 after 4 days (t-test, 0.0041; F-test, ns). When paired with cultures of *T. harzianum* on bCA, the growth of *A. bisporus* was unaffected (Table 3.5).

The presence of *A. bisporus* on DWA, though spatially separated, had a significant inhibitory effect on the growth of *T. harzianum* strain Th1(c) at all data collection times. In contrast, *T. harzianum* strain T7 (Th2) was unaffected by the presence of *A. bisporus* on DWA. The growth of *T. harzianum* was unaffected by *A. bisporus* on MEA (Table 3.6). *T. harzianum* strain T7 was also unaffected by the presence of *A. bisporus* on fCA (Table 3.6). Strain Th1(c) was significantly inhibited by *A. bisporus* but only after 3 days incubation (t-test, P = 0.0025; F-test, ns). On bCA *T. harzianum* strains TD15 and Th2F were not influenced by the presence of *A. bisporus* (Table 3.6). *A. bisporus* appeared to have an inhibitory effect towards *T. harzianum* strain Th1(c), which was significantly different after 1 (t-test, P = 0.002; F- test, ns) and 2 days (t-test, P = 0.011; F-test, ns) incubation. *T. harzianum* strain T7 was significantly inhibited by *A. bisporus* on bCA after 1 (t-test, P = 0.0047; F-test, ns) and 3 days (t-test, P = 0.0068; F-test, ns)

Table 3.5. Comparison of colony growth of *A. bisporus* in pure cultures and paired, in a closed environment, opposite a culture of *T. harzianum* to investigate the production of possible volatile antibiotic compounds.

Medium	Time (days)	Growth of *A. bisporus* (mean diameter (mm) ± SEM)				
Isolate pairings		A12	A12 α Th1(c)	A12 α TD15	A12 α T7	A12 α Th2F
DWA	1	18 ± 0	17 ± 0		17± 0	
	2	21 ± 1	20 ± 0		20± 0	
	3	23 ± 0	22 ± 0*		23 ± 0	
	4	26± 0	24 ± 0*		25± 0	
MEA	1	20 ± 0	21 ± 0		21± 0†	
	2	22± 0	24± 0†		24± 1†	
	3	26 ± 1	27 ± 0		28 ± 0†	
	4	27± 1	29± 1		30± 0††	
fCA	1	24 ± 2	26 ± 4		28± 2	
	2	30± 1	35 ± 2†		33± 2	
	3	36 ± 1	41 ± 3		39± 2	
	4	39± 1	46 ± 2†		46 ± 1†	
bCA	1	18 ± 1	16± 1	17± 1	18± 1	21 ± 1
	2	23 ± 2	20± 2	21± 2	24 ± 2	22± 1
	3	26± 2	24 ± 2	25 ± 2	26 ± 2	27 ± 1
	4	29 ± 2	29± 3	31 ± 2	30 ± 1	31 ± 1

† Indicates *A. bisporus* growth was significantly higher than the control, according to unpaired, two-tailed t-tests (P<0.05). †† Indicates that *A. bisporus* growth was significantly higher than control according to t-test and F-test (<0.05).

* Indicates *A. bisporus* growth was significantly lower than the control, according to unpaired, two-tailed t-tests (P<0.05). ** Indicates that *A. bisporus* growth was significantly lower than control according to t-test and F-test (<0.05).

Table 3.6. Growth of *T. harzianum* in pure cultures and paired, in a closed environment, opposite a culture of *A. bisporus*.

Medium	Time (days)	Growth of *T. harzianum* (mean diameter (mm) ± SEM)							
Isolate pairings		Th1(c)	Th1(c) α A12	TD15	TD15 α A12	T7	T7 α A12	Th2F	Th2F α A12
DWA	1	28± 1	25± 0*			27 ± 0	27± 0		
	2	62 ± 0	55± 1*			59± 1	60 ± 0		
	3	84± 0	79± 1**			83 ± 3	83± 0		
MEA	1	29 ± 1	28 ± 1			28 ± 0	27± 1		
	2	67 ± 1	65± 1			65± 1	64± 2		
	3								
fCA	1	15± 0	15± 1			24± 0	24 ± 1		
	2	49 ± 1	45 ± 1			53± 1	57 ± 3		
	3	84± 1	76± 1*			83± 2	84 ± 1		
bCA	1	22 ± 1	18 ± 1*	14 ± 1	16 ± 1	22± 1	19± 0*	19± 1	17± 2
	2	46± 2	40 ± 1*	41± 2	45 ± 1	48 ± 1	45 ± 1	40± 0	40± 3
	3	70± 2	67± 1	71± 3	77 ± 2	75 ± 1	68± 1*	66 ± 2	63 ± 5

† Indicates *T. harzianum* growth was significantly higher than the control, according to unpaired, two-tailed t-tests (P<0.05). †† Indicates that *T. harzianum* growth was significantly higher than control according to t-test and F-test (<0.05).

* Indicates *T. harzianum* growth is significantly lower than the control, according to unpaired, two-tailed t-tests (P<0.05). ** Indicates that *T. harzianum* growth was significantly lower than control according to t-test and F-test (<0.05).

T. harzianum genotype 1: Th1(c) and TD15 and genotype 2: T7 and Th2F.

incubation (Table 3.6).

Differences were recorded between *T. harzianum* strains and their effects on the growth of *A. bisporus* under the conditions of double plate system. However, comparisons of the differences of the effects of *T. harzianum* strains on the growth of *A. bisporus* using one-way ANOVA indicated that there were no significant differences (see Appendix). Identical analyses were performed on the differences between controls and double plated *T. harzianum* strains. No significant difference was found between the *T. harzianum* strains except however, on bCA after 2 days incubation ($P = 0.048$). The value of P indicates that these results were only just significantly different and as such, Bonferroni's multiple comparison test failed to distinguish any significant differences between strains (see Appendix).

In short, Th1 was inhibitory towards *A. bisporus* on DWA and genotypes 1 and 2 exhibited stimulatory effects towards *A. bisporus* on MEA and fCA. The growth of *A. bisporus* was unaffected on bCA. In contrast, Th1 was inhibited by *A. bisporus* on all media except MEA (Th1(c)) and bCA (TD15). Th2 exhibited no detectable effects in the presence of *A. bisporus* with the exception of strain T7 on bCA.

3.1.2 Study of interactions between A. bisporus and T. harzianum inoculated in compost – a scale model of the green mould problem in the commercial mushroom industry

Investigations into the possible modes of interactions between *T. harzianum* and *A. bisporus* on nutrient agar provided limited suggestions as to the nature of the interaction. Therefore a model system using screw-capped plastic tubes was adapted for further investigations (Seaby 1987; Grogan and Gaze 1995; Fletcher 1997). This model had the advantage of being flexible and several adaptations were made to investigate various possible mechanisms. Mushroom spawn (A12) and compost (Formula 8) were both supplied by the mushroom unit at HRI Wellesbourne. *T. harzianum* inoculum was collected from sporulating cultures (Materials and Methods 2.0.2).

Assessments of fungal colonisation of compost in the tubes were made on a visual scoring system, previously established (Grogan H., HRI Wellesbourne, pers. comm.). *A. bisporus* growth was assessed on a scale of 0 to 3: 0 – representing no visible colonisation to 3 – indicating fully colonised compost, observed as a dense, white,

characteristic mycelium. *T. harzianum* colonisation was measured as the distance (mm) from initial inoculum source at which sporulation was observed and, due to variation in the amount of sporulation, an additional score for the intensity of the sporulation was given: +, little sporulation to +++, for profuse sporulation. Assessments began with the selection of example tubes, which corresponded to the scale of the *A. bisporus* scoring system. These selected tubes were then used to compare with other tubes, which ensured consistency was maintained within the scoring system.

3.1.2(i) The effect of T. harzianum inoculum source on the growth of A. bisporus in compost

In order to determine the requirement of *T. harzianum* for an initial nutrient source, *T. harzianum* inoculum was added on a variety of bases (Table 3.7).

Agaricus bisporus inoculum (spawn grain) was also added to either the bottom of tube ("adjacent") together with *T. harzianum* inoculum, or to the top of the tube ("separate") separated spatially from *T. harzianum* inoculum. The inoculum positions allowed assessment of possible antagonism at distance. Compost (*ca.* 30g) was added and compressed by hand. The caps were opened a quarter turn and tubes were incubated at 25°C for 3 weeks. Controls of the individual inocula were produced. Five replicates were produced for each treatment.

Inoculation of *T. harzianum* strain T7 (Th2) as a spore suspension had different effects dependent on the combination of filter paper and glucose solution with the spore suspension (Table 3.8). The growth of *A. bisporus* was not affected by any of the spore suspension treatments when *A. bisporus* inoculum was spatially separated from the *T. harzianum* spore suspension (Table 3.8). However, when placed close to *T. harzianum* inoculum, the growth of *A. bisporus* was visibly reduced compared to the control. This difference was only significant for treatment 1 (addition of spore suspension alone) where growth of *A. bisporus* in tubes with 'adjacent' inocula differed significantly to that in the control tubes and in those tubes with inoculum separated spatially (Table 3.8).

Trichoderma harzianum exhibited increased growth in tubes where *A. bisporus* was

Table 3.7. Types of inoculum base used for *T. harzianum* spores.

Inoculum Base	Method
Spore suspension (10^7 spores ml^{-1})	1) 100µl added directly 2) 100µl on sterile filter paper 3) 100µl on sterile filter paper + 100µl 1% (w/v) glucose
Sterile rye grain	Rye grain rolled in sporulating culture
Spawn grain	Spawn rolled in sporulating culture

Table 3.8. Effects of the addition of *T. harzianum* as a spore suspension on the growth of *A. bisporus* and *T. harzianum* in mushroom compost.

Inoculum base*	*A. bisporus* growth+ (mean ± SEM)			*T. harzianum* growth† (mean ± SEM)		
	Control	Separate**	Adjacent	Control	Separate	Adjacent
1	3 ± 0.0^{a}	3 ± 0.0^{a}	2.4 ± 0.245^{b}	10± 10.0	0.0	12± 5
2	3 ± 0.0	3 ± 0.0	2.6 ± 0.245	0.0	0.0	16 ± 10
3	3 ± 0.0	3 ± 0.0	2 ± 0.0	0.0^{c}	0.0^{c}	12 ± 5^{d}

*Inoculum base: 1) 100µl (10^7 spores ml^{-1} suspension) of *T. harzianum* strain T7 (Th2) added to bottom of tube; 2) 100µl (10^7 spores ml^{-1} suspension) of *T. harzianum* strain T7 added to sterilised filter paper placed at bottom of the tube; 3) 100µl (10^7 spores ml^{-1} suspension) of *T. harzianum* strain T7 added to sterilised filter paper with 100µl glucose solution (1% w/v).

+ Growth assessed visually on a 0 to3 scale.

† Growth assessed as distance (mm) from the source of inoculum.

** Inocula positions: 'separate', inocula placed at opposite ends of tube; 'adjacent', inocula placed together at bottom of tube.

Values followed by different letters are significantly different according to one-way ANOVA ($P<0.05$).

Table 3.9. The effect of the addition of *T. harzianum* spores on sterilised rye grain towards the growth of *A. bisporus* in mushroom compost.

Position of spawn*	Growth of *A. bisporus* strain A12 (mean ± SEM)				
Isolate pairings	Control	A12 α Th1(c)	A12 α TD15	A12 α T7	A12 α Th2A
Separate	3.0	2.8 ± 0.2	2.8 ± 0.2	2.6 ± 0.245	2.6 ± 0.245
Adjacent	3.0^{a}	2.2 ± 0.2^{a}	2.2 ± 0.583^{ac}	1.0 ± 0.633^{bc}	0.0^{b}

* Inocula positions – see Table 3.8. Values followed by different letters are significantly different according to one-way ANOVA ($P<0.05$). Growth assessed as on Table 3.8 legend.

T. harzianum strains: Th1(c) and TD15 (Th1) and T7 and Th2A (Th2).

added to the bottom of the tube along with the *T. harzianum* inoculum (Table 3.8). *T. harzianum* appeared to be unable to grow when separated spatially from *A. bisporus* inoculum. *A. bisporus* at the bottom of the tube in treatment 3 (spore suspension and 1% glucose) significantly enhanced the growth of *T. harzianum* which was above that of controls and in those tubes in which inocula were separated (Table 3.8).

The introduction of *T. harzianum* spores on sterilised rye grain had no significant effect on the growth of *A. bisporus* when the two fungi were inoculated at opposite ends (Table 3.9). However Th2 appeared to reduce the growth of *A. bisporus* more than did the non-aggressive genotype (strains Th1(c) and TD15) (Table 3.9). The growth of *A. bisporus* in tubes where inocula were adjacent appeared to be inhibited (cf. controls) regardless of *T. harzianum* strain (Table 3.9). Non-aggressive strains of *T. harzianum* exhibited a slight inhibitory effect towards *A. bisporus* but this was not significantly different to the controls. The aggressive *T. harzianum* strains significantly reduced the growth of *A. bisporus* when inocula were adjacent to one another, as opposed to higher *A. bisporus* growth in *A. bisporus* controls and tubes where inocula were separated (Table 3.9).

Growth of non-aggressive *T. harzianum* isolates (Th1(c) and TD15) from rye grain inoculum appeared to be limited in both control and treatment tubes, however more growth occurred in control tubes and in those with inocula adjacent than those with inocula separated. Strain Th1(c) grew significantly less in the presence of *A. bisporus* compared to the Th1(c) alone control tubes (Table 3.10). Control tubes of aggressive *T. harzianum* (strains T7 and Th2A) and treatments with adjacent inocula demonstrated substantial colonisation that was significantly higher than in tubes with inocula separated (Table 3.10). In addition, the growth of Th2 was enhanced by the close proximity of spawn grain and was significantly higher than in treatments with inocula separated. There was no significant difference in colonisation by aggressive genotypes between the controls and those tubes with inocula adjacent (Table 3.10). The measure of growth of *T. harzianum* was corrected against the control (treatment value was subtracted from the control) to allow a comparison between strains (Table 3.11). Comparisons of the controls revealed significant differences between genotypes 1 and 2; the latter displayed substantially higher colonisation of mushroom

Table 3.10. Effects of the position of *A. bisporus* spawn grain on the growth of *T.harzianum* introduced to mushroom compost on sterilised rye grain.

T. harzianum strain	Growth of *T. harzianum* (mean ± SEM)		
Treatment	Control	Separate*	Adjacent
Th1(c)	15 ± 2^{a}	5^{b}	8 ± 2^{b}
TD15	9 ± 1	6 ± 1	10 ± 2
T7	42 ± 8^{c}	6 ± 1^{d}	30 ± 7^{c}
Th2A	50^{e}	19 ± 8^{f}	50^{e}

* Inocula positions – see Table 3.8 legend. Growth assessed as Table 3.8 legend. *T. harzianum* genotypes – see Table 3.9 legend. Values followed by different letters (in rows) are significantly different according to one-way ANOVA ($P<0.05$).

Table 3.11. Comparison of the growth of *T. harzianum* strains from a rye grain inoculum base in compost inoculated with *A. bisporus* spawn in different positions.

T. harzianum strain	Growth of *T. harzianum* (mean ± SEM)		
Treatment	Control	Separate*	Adjacent**
Th1(c)	15 ± 2^{a}	10 ± 2^{ce}	8 ± 2
TD15	9 ± 1^{a}	2 ± 1^{c}	-1 ± 3
T7	42.4 ± 8^{b}	37 ± 8^{d}	4 ± 16
Th2A	50^{b}	31 ± 8^{de}	0

* The values represent the difference between the control and treatment (C-T) value given in table 3.10. Inocula positions – see Table 3.8 legend.

** The values represent the difference between control and treatment (C-T) value (Table3.10). Growth assessed as on Table 3.8 legend. Genotypes as described on Table 3.9 legend. Values followed by different letters (in columns) are significantly different according to one-way ANOVA ($P<0.05$).

compost than the former. The corrected measurements for separated inocula revealed significant differences between genotypes 1 and 2 with the latter displaying greater colonisation. No significant difference between genotypes was found in *T. harzianum* colonisation when inocula were adjacent.

The presence of *T. harzianum* on spawn grain visibly reduced the growth of *A. bisporus* (Table 3.12). The aggressive genotype exhibited a substantial inhibitory effect towards *A. bisporus* compared to the controls and to non-aggressive *T. harzianum* isolates, however this was not statistically significant. The measurements of *A. bisporus* growth were corrected against the controls (by subtracting the treatment value from control value) and the effects of *T. harzianum* isolates were compared (Table 3.12). One-way ANOVA revealed significant difference ($P = 0.0266$) between the inhibition of *A. bisporus* by isolates of *T. harzianum*, however a multiple comparison test to compare all pairings revealed no significant differences. Growth of *A. bisporus* was inhibited by aggressive strains of *T. harzianum* more than by non-aggressive isolates.

The growth of *T. harzianum* in the presence of *A. bisporus* exhibited no difference to that in the control tubes (Table 3.13). In the absence (control) and presence of *A. bisporus* the aggressive genotype of *T. harzianum* (strains T7 and T32) achieved substantially higher colonisation than did the non-aggressive genotype (Table 3.13). Measurements of *T. harzianum* growth were corrected against the controls and were found not to differ significantly to one another.

In summary the inoculum base appeared to affect the colonisation of compost by *T. harzianum*. Spore suspensions of *T. harzianum* added alone or in combination with filter paper and glucose solution had little effect on *A. bisporus* growth. This inoculum base also produced limited *T. harzianum* growth in the presence or absence of *A. bisporus*. Inoculation of Th2 spores on rye grain greatly reduced *A. bisporus* growth. In contrast, the growth of Th2 was high in the absence of *A. bisporus* and in tubes where inocula were adjacent. The introduction of *T. harzianum* spores on spawn grain reduced the growth of *A. bisporus*. The aggressive Th2 (strains T7 and Th2A) exhibited a greater inhibitory effect on *A. bisporus* than did non-aggressive Th1 (strains Th1(c) and TD15). *T. harzianum* compost colonisation from spawn inoculum base was no different

Table 3.12. Effects of the introduction of *T. harzianum* spores on spawn grain towards the growth of *A. bisporus* in mushroom compost.

Value	Growth of *A. bisporus* (mean ± SEM)				
Isolate pairings	Control	A12 α Th1(c)	A12 α TD15	A12 α T7	A12 α T32
mean ± SEM	3.0 ± 0.0	2.8 ± 0.2	2.4 ± 0.245	1.2 ± 0.5831	1.2 ± 0.49
difference* (mean ± SEM)		0.2 ± 0.2	0.6 ± 0.245	1.8 ± 0.583	1.8 ± 0.49

* This value represents the difference between control and treatments (C-T) to allow comparisons between treatments.

Growth assessed as on Table 3.8 legend. *T. harzianum* strains: Th1(c) and TD15 (genotype1) and T7 and T32 (Th2).

One-way ANOVA revealed significant difference between the treatment differences (P = 0.0266), however statistical analysis between individual pairings using Bonferroni's multiple comparison test found no significant difference.

Table 3.13. Growth of *T. harzianum* inoculated on spawn grain in mushroom compost in the presence of *A. bisporus*

Treatment*	Growth of *T. harzianum* (mean ± SEM)			
	Th1(c)	TD15	T7	T32
Control	3± 1[a]	7± 1[ab]	28 ± 10[ab]	35± 9[b]
A. bisporus	5	9 ± 3	30± 12	30± 9
Difference	-2± 1	-2 ± 4	-2 ± 9	5± 15

* Treatments included: 1) control, no *A. bisporus*; 2) *A. bisporus*, spawn grains present; 3) value represents the difference between treatments 1 and 2. Growth assessed as on Table 3.8 legend. Genotypes described on Table 3.12 legend. Values followed by different letters (in rows) are significantly differently according to one-way ANOVA ($P<0.05$).

in the presence of *A. bisporus* to the *T. harzianum* controls. Aggressive Th2 exhibited significantly higher growth than did Th1.

Inoculum position was investigated by either separating the inocula spatially or placing inocula adjacent to one another and generally *A. bisporus* was inhibited more when inocula were adjacent than when separated. In addition, *T. harzianum* displayed greater growth when inocula were adjacent than when separated.

3.1.2(ii) Effects of different T. harzianum genotypes on A. bisporus growth in compost

Plastic tubes (60ml capacity) filled with unspawned compost (30g fresh weight) were inoculated with both *T. harzianum* and *A. bisporus*. Approximately 100 spawn grains were added to a profusely sporulating culture of *T. harzianum* and agitated for 30 seconds until the white spawn grains were covered in green conidia. Three *A. bisporus* spawn grains and *T. harzianum* inoculum (3 grains) were placed in the bottom of a sterile tube and were subsequently covered with compost (compressed by hand). The lids were opened a quarter turn to simulate semi-anaerobic conditions. Tubes were incubated at 25°C, in the dark with high relative humidity (*ca.* 80%) for 3 weeks. Controls of the individual fungi were assembled. Ten replicates were produced for each treatment as substantial variation had occurred previously.

The growth of *A. bisporus* was substantially inhibited in the presence of aggressive strains of *T. harzianum* (T7 and T32) (Table 3.14). The inhibitory effect on *A. bisporus* caused by *T. harzianum* strains T7 and T32 was significantly different when compared to the controls and the non-aggressive *T. harzianum* isolates (Table 3.14). This difference was also apparent when the growth measurements in the presence of *T. harzianum* were corrected against the *A. bisporus* controls (by subtracting the treatment value from the control). Aggressive *T. harzianum* had an apparent inhibitory effect, while non-aggressive isolates failed to have any effect on *A. bisporus* growth.

Colonisation of mushroom compost by isolates of *T. harzianum* differed considerably; however the differences were consistent whether *A. bisporus* was present or absent (Table 3.15). In the controls and in the presence of *A. bisporus*, aggressive strains of *T.*

Table 3.14. Growth of *A. bisporus* in the presence of *T. harzianum* introduced to compost on spawn grain.

Value	Growth of *A. bisporus*				
Isolate pairings	Control	A12 α Th1(c)	A12 α TD15	A12 α T7	A12 α T32
mean ± SEM	2.8 ± 0.133^{a}	2.8 ± 0.133^{a}	2.7 ± 0.153^{a}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}
difference* (mean ± SEM)		0.0 ± 0.211^{c}	0.1 ± 0.18^{c}	2.8 ± 0.133^{d}	2.7 ± 0.213^{d}

* Treatment measurements were corrected against the control and are represented as the difference between the controls and treatment.

Growth assessed as on Table 3.8 legend. Genotypes as described on Table 3.12 legend.

Values followed by different letters (in rows) are significantly different according to one-way ANOVA ($P<0.05$).

Table 3.15. The effect of the presence of *A. bisporus* on the growth of *T. harzianum*.

Treatment*	Growth of *T. harzianum* (mean ± SEM)			
	Th1(c)	TD15	T7	T32
1) Control	7 ± 1^{a}	6 ± 1^{a}	49 ± 2^{b}	46 ± 3^{b}
2) α *A. bisporus*	4 ± 1	7 ± 1	50	47± 2
3) difference	3 ± 1	-1 ± 1	-1± 2	-1± 2

* The treatments were: 1) control, no *A. bisporus*; 2) *A. bisporus* added as spawn grain; 3) treatment values were corrected against the controls by taking the difference between controls and treatment 2.

Growth assessed as on Table 3.8 legend. Genotypes as described on Table 3.12 legend.

Values followed by different letters are significantly different according to the Friedman test ($P<0.05$) for non-parametric data.

harzianum achieved substantially more growth in compost than non-aggressive isolates. There was no significant difference between growth in *T. harzianum* controls and that when exposed to *A. bisporus*; however in the presence of *A. bisporus*, aggressive *T. harzianum* exhibited slightly more growth (Table 3.15). Comparison of the controls using the Friedman test for non-parametric data revealed significant differences between Th1 (non-aggressive) and 2 (aggressive). *T. harzianum* growth measurements in the presence of *A. bisporus* were corrected against the control values and subjected to one-way ANOVA, which revealed no significant differences. These results are representative of three separate experiments.

To summarise, *A. bisporus* was inhibited significantly by Th2 (strains T7 and T32) and was unaffected by Th1 (strains Th1(c) and TD15). Th2 exhibited extensive growth in compost in the presence (treatment) or absence (control) of *A. bisporus*, while Th1 produced limited growth in both treatment and control tubes.

3.1.2(iii) Comparison of growth of T. harzianum in compost in the presence of viable or non-viable A. bisporus spawn grains

Previous reports suggested an obligate-parasitic relationship between *T. harzianum* and *A. bisporus* (Seaby, 1987); in contrast, data from various tube tests in this work revealed colonisation of compost by *T. harzianum* in the absence of *A. bisporus*. *T. harzianum* spores inoculated on *A. bisporus*-infested spawn grains may possibly survive in compost because of the presence of *A. bisporus* mycelium on the grain. To investigate whether live *A. bisporus* was necessary for substantial colonisation, *T. harzianum* was also inoculated on *A. bisporus* spawn grain that had been sterilised.

Initially, several techniques were used in an attempt to sterilise *A. bisporus* spawn and render the mycelium non-viable. The techniques included, i) exposure to propylene oxide for 18 hours, ii) gamma irradiation for 48 hours, iii) high power microwaving for 2.5 minutes and iv) autoclaving at 121°C for 20 minutes. A sample of the sterilised grains was plated on MA and incubated at 25°C to ensure non-viability. Growth was only detected from the irradiated spawn. Heating in a microwave oven was the chosen method of *A. bisporus* spawn grain sterilisation as it was rapid and appeared not to increase the moisture content of the spawn, unlike autoclaving.

The sterilised spawn was rolled in *T. harzianum* spores for the *T. harzianum* alone control and live spawn was used as a base for *T. harzianum* inoculum in the interaction tubes inoculated with *A. bisporus*.

The *T. harzianum* spore inoculum was unknown and could have varied between experiments therefore the number of spores was quantified. This was achieved by removing a sample of 10 spawn grains previously rolled in *T. harzianum* spores. The sample was placed in a sterile, plastic 9ml universal tube with 2ml of 0.04% Tween 80 to dislodge the attached spores. The tube was inverted 5 times and the resulting suspension was filtered through 2 layers of muslin prior to the calculation of spore load.

Viable spawn grain was capable of carrying a higher spore load than non-viable spawn grain (Table 3.16). Significant differences occurred for Th2 spore loads when viable and non-viable inocula were compared, e.g. 6.46 and 6.803 for T7 non-viable and viable spawn grain respectively.

T. harzianum mycelial growth was higher from the non-viable spawn inoculum base than from the viable spawn and this difference was significant for strains Th1(c), KPNT, Th2A and Th2F (Table 3.17). Comparisons between *T. harzianum* strains revealed genotypic differences; aggressive strains T7 and Th2A demonstrated significantly higher growth than Th1 strains and aggressive strains, KPNT and Th2F from the non-viable inoculum base (Table 3.17). *T. harzianum* growth from a viable spawn inoculum base also exhibited differences. Th2 strain T7 displayed significantly higher growth than that of non-aggressive strains Th1(c) and TD15 and aggressive strains KPNT and Th2F. In addition a genotype 3 strain, TD7 (also non-aggressive) and strain Th2A (Th2) exhibited higher growth than strain Th2F (Th2).

In summary, viable spawn grain carried higher *T. harzianum* spore loads than did non-viable spawn. Also more *T. harzianum* growth occurred from non-viable spawn than from viable spawn. Two aggressive strains (T7 and Th2A) demonstrated significantly higher growth than other strains from non-viable spawn. In contrast two aggressive strains (T7 and Th2A) and a non-aggressive (TD7) produced higher growth from viable

Table 3.16. Quantification of *T. harzianum* spore load on viable and non-viable spawn grain.

Spawn	Number of *T. harzianum* spores per spawn grain (log_{10}) (mean + SEM)							
Isolates	Th1(c)	TD15	T28JF	TD7	T7	KPNT	Th2A	Th2F
non-viable	$6.68 \pm 0.095^{*a}$	6.54 ± 0.029^{ab}	6.4 ± 0.034^{ab}	$6.46 \pm 0.078^{*ab}$	$6.35 \pm 0.012^{*bc}$	$6.38 \pm 0.01^{*abc}$	$6.45 \pm 0.027^{*ab}$	6.06 ± 0.111^{c}
viable	$6.9 \pm 0.003^{*d}$	6.66 ± 0.234^{de}	6.67 ± 0.093^{de}	$6.803 \pm 0.02^{*d}$	$6.69 \pm 0.007^{*d}$	$6.85 \pm 0.061^{*d}$	$6.99 \pm 0.032^{*d}$	6.31 ± 0.078^{e}

* Indicates significant difference in spore load for individual *T. harzianum* strains between viable and non-viable spawn grain inoculum base (i.e. in columns). Growth assessed as on Table 3.8 legend. Genotype 1: Th1(c), TD15 and T28JF; genotype 3: TD7 and genotype 2: T7, KPNT, Th2A and Th2F. Values (in rows) followed by different letters are significantly different according to one-way ANOVA and Bonferroni's multiple comparison test.

Table 3.17. Growth of *T. harzianum* in mushroom compost from an inoculum base of viable or non-viable spawn.

Treatment	Growth of *T. harzianum* (mean ± SEM)							
Isolate	Th1(c)	TD15	T28JF	TD7	T7	KPNT	Th2A	Th2F
non-viable	$8.7 \pm 0.50^{*a}$	8.6 ± 1.056^{a}	7.5 ± 3.408^{a}	22.2 ± 3.844^{ab}	50.0 ± 0.0^{b}	$9.5 \pm 0.934^{*a}$	$46.5 \pm 3.5^{*b}$	$7.4 \pm 0.267^{*a}$
viable	$3.8 \pm 1.365^{*ce}$	5.1 ± 1.354^{ce}	6.7 ± 1.096^{cde}	23.5 ± 4.23^{cd}	45.0 ± 5.0^{d}	$4.3 \pm 1.844^{*ce}$	$21.1 \pm 5.429^{*cd}$	$1.6 \pm 0.846^{*e}$

* Indicates significant difference (in columns) between the growth of *T. harzianum* strain in compost from viable and non-viable spawn according to two-tailed, upaired t-test (Gaussian data) and Mann Whitney test (non-parametric data) ($P<0.05$). Growth assessed as on Table 3.8 legend. Values followed by different letters (in rows) indicate significant difference according to Friedman test for non-parametric data and Dunn's multiple comparison test ($P<0.05$). Genotype 1: Th1(c), TD15, T28JF; genotype 3: TD7 and genotype 2: T7, KPNT, Th2A and Th2F.

spawn than the other strains from viable spawn.

3.1.2(iv) Investigation of volatile products of A.bisporus and T.harzianum in compost

To investigate any possible reciprocal effects of volatile products of *A. bisporus* and *T. harzianum* a double tube system was developed in which the fungi were inoculated in tubes filled with compost that were separated by a physical barrier. Porous membranes were used to separate the fungi but not low molecular weight products. The apparatus consisted of two tubes attached by the lids, which had a circular area removed (5 mm less than the diameter of the lid). Porous membranes were placed between the two lids, which were then taped together (Fig 3.1). The apparatus was then sterilised by gamma irradiation for 72 hours.

A range of materials was chosen as membranes to permit transmission of volatiles but not allow the passage of either *T. harzianum* or *A. bisporus*. These were: nitrocellulose membrane filters of various pore size (0.1, 0.8 and 8.0 μm), dialysis tubing and membranes used for molecular studies Hybond-N and Hybond-N^+. The permeability of these membranes with respect to gas was investigated. Membranes were loaded into a nalgene filter and attached to a syringe full of air, the filter container was submerged in water and the air was slowly pushed through. Air bubbles were seen after air had passed through the filters and therefore all membranes were determined gas permeable. To ensure that the double tube assembly was gas permeable a hole was made in the base of each tube using a hot cork borer. A syringe was inserted in one of the holes, sealed with parafilm and the whole assembly was submerged in water. Air from the syringe was pushed through the system and was observed to escape only from the hole in the lower tube. Hence the apparatus was determined as gas permeable and capable of allowing the passage of low molecular weight volatile compounds through the membrane.

Fungal inoculum was placed either side of the membrane or at distance from each other at the bottom of each tube (Fig 3.1). Compost (*ca.* 30 g) was added to the tubes and compressed by hand. The tubes were incubated at 25°C in the absence of light and with high relative humidity (*ca.* 80%) for three weeks. *T. harzianum* strain T7 (Th2) was used

Figure 3.1. Apparatus to investigate possible production of volatile compounds produced by *T. harzianum* and *A. bisporus.*

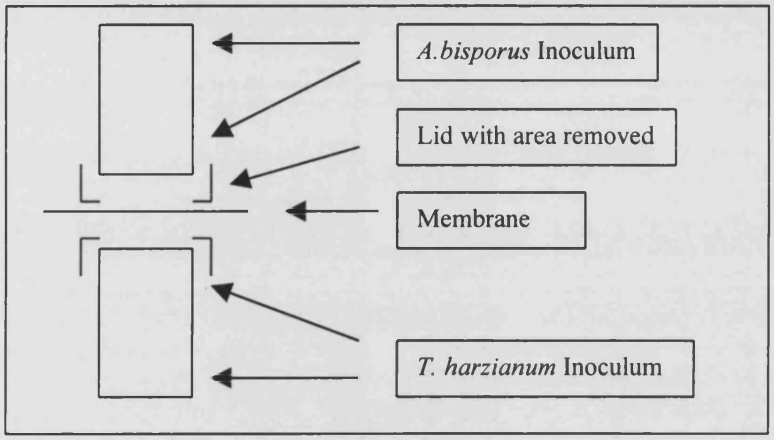


Table 3.18. The growth of *A. bisporus* in the presence of, though physically separated from, *T. harzianum* in compost.

Membrane[†]	*A. bisporus* growth (mean ± SEM)	
	Close[a]	Apart[a]
Control	3.0 ± 0.0*	3.0 ± 0.0
nitrocellulose 0.1μm[b]	3.0 ± 0.0*	3.0 ± 0.0
nitrocellulose 0.8μm	2.0 ± 0.0*	2.667 ± 0.333
nitrocellulose 8.0μm	1.667 ± 0.8819*	3.0 ± 0.0
dialysis tubing	1.667 ± 0.333*	3.0 ± 0.0
hybond-N[b]	3.0 ± 0.0*	3.0 ± 0.0
hybond-N^{+b}	3.0 ± 0.0*	3.0 ± 0.0
no membrane	1.33 ± 0.333*	nd

[†] Membranes with various pore sizes were chosen in an attempt to separate physically the two fungi but to allow the passage of low molecular weight volatile compounds. Controls were set up that contained *A. bisporus* only. Replicates with no membranes allowed direct contact between fungi. Growth as assessed on Table 3.8 legend.

[a] Represents the position of the inocula, close: inocula placed either side of membrane; apart: inocula at opposite ends of tubes.

[b] Indicates the presence of intact membranes.

* Indicates significant difference between treatments and the effect on *A. bisporus* growth (in columns) according to the Friedman test for non-parametric data ($P<0.05$). However Dunn's multiple comparison test to compare all individual pairings of treatments indicated no significant difference.

nd – not determined.

Table 3.19. The growth of *T. harzianum* in compost in the presence of, though physically separated from, *A. bisporus*.

Membrane[†]	Growth of *T. harzianum* (mean ± SEM)	
	Close[a]	Apart[a]
Control	100 ± 0.0	100 ± 0.0
nitrocellulose 0.1μm	36.67 ± 13.33	50.0 ± 0.0
nitrocellulose 0.8μm	56.67 ± 23.33	50.0 ± 0.0
nitrocellulose 8.0μm	40.0 ± 0.0	5.0 ± 2.887[*]
dialysis tubing	66.67 ± 16.67	47.67 ± 2.33
hybond-N	36.67 ± 13.33	50.0 ± 0.0
hybond-N[+]	46.67 ± 3.33	50.0 ± 0.0
no membrane	100 ± 0.0	nd

[†] Membranes as described on Table 3.18 legend. [a] Inoculum position as described on Table 3.18 legend. Growth assessed as on Table 3.8 legend – values beyond 50 indicate growth of *T. harzianum* into the *A. bisporus* tube as tubes are only 50 mm in height. [*] value is significantly different to other values in column according to one-way ANOVA ($P<0.05$).

nd – not determined.

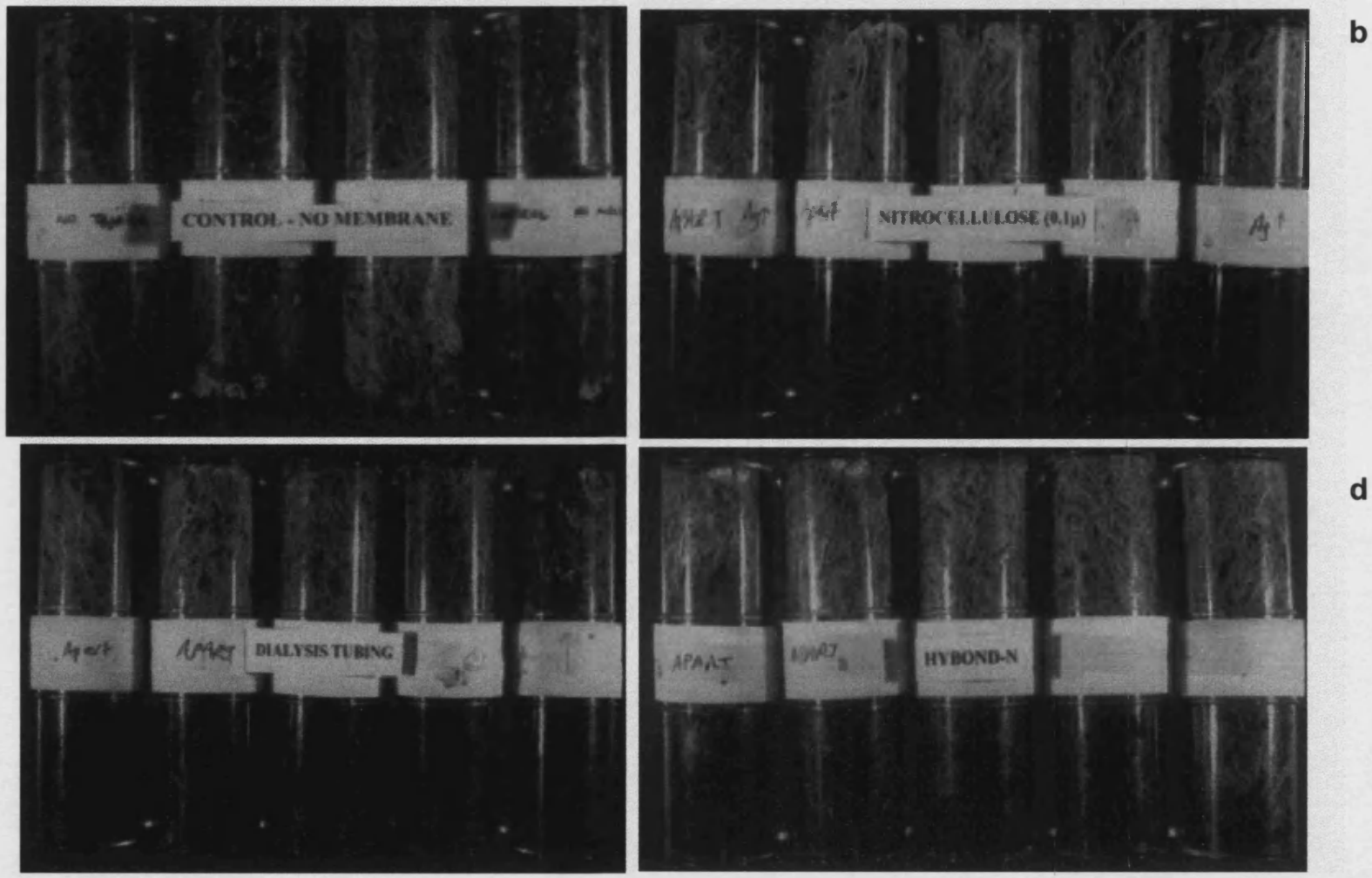


Figure 3.2: The growth of *A. bisporus* and *T. harzianum* in compost separated by a gas permeable membrane. (a) Control tubes with no membrane. (b) Nitrocellulose - pore size 0.1 µm. (c) Dialysis tubing. (d) Hybond-N membrane.

for this study.

Assemblies with inocula in close proximity and with no membrane displayed reduced growth of *A. bisporus* in the presence of *T. harzianum,* which grew into the tube in which *A. bisporus* was inoculated (Table 3.18, Table 3.19 and Fig. 3.2a). Similarly, *A. bisporus* growth was reduced by *T. harzianum* in assemblies where the membrane was damaged (Table 3.18). Nitrocellulose membranes with 0.8 and 8.0 μm pore size were very brittle and therefore easily damaged, whereas dialysis tubing (Fig. 3.2c) was rapidly degraded presumably by cellulases of *A. bisporus* and/or *T. harzianum* origin. Membranes that remained intact included nitrocellulose (0.1 μm) (Fig. 3.2b), Hybond-N (Fig. 3.2d) and Hybond-N^+. In these assemblies, *A. bisporus* exhibited maximal growth equivalent to that in control tubes, regardless of the substantial *T. harzianum* colonisation in the opposite tube (Table 3.18 and 3.19). The growth of *A. bisporus* with different membranes was significantly different using the Friedman test ($P = 0.0146$).

Positioning the inocula at opposite ends of the different tubes with a larger distance between them had the apparent effect of reducing the inhibitory effect of *T. harzianum* in tubes with damaged membranes (Table 3.18). *A. bisporus* growth was maximal for all membranes except with nitrocellulose (8.0 μm) when growth was slightly reduced. *A. bisporus* growth was substantial in the presence of either intact or damaged membranes when inocula were separated at distance (Table 3.18).

In close proximity to *A. bisporus* inoculum, *T. harzianum* exhibited substantial colonisation of compost when membranes were damaged (Table 3.19). The growth of *T. harzianum* in assemblies with intact membranes differed depending on the inocula positions. At distance, *T. harzianum* growth was higher than that in close proximity to *A. bisporus* (Table 3.19). Th2 in assemblies without membranes and in the absence of *A. bisporus* colonised both tubes. When inocula were placed apart, *T. harzianum* growth was slightly higher than in tubes with inocula in close proximity. There were two exceptions to this, with the dialysis tubing membrane *T. harzianum* displayed reduced growth and with nitrocellulose (8.0μm) there was significantly reduced colonisation (Table 3.19).

To summarise, *A. bisporus* was only inhibited in the absence of a membrane or when a membrane was damaged and thus allowed contact between the two fungi (Table 3.18). This reduction in *A. bisporus* growth was only observed when inocula were in close proximity; when inocula were separated at distance, *A. bisporus* was unaffected. *T. harzianum* displayed substantial growth in the presence or absence of a membrane; typically growth was 2-fold higher in the absence of a membrane and was unaffected by the inocula positions (Table 3.19). In assemblies with intact membranes (nitrocellulose 0.1 μm, Hybond-N and Hybond-N^+) *T. harzianum* appeared to be slightly inhibited by *A. bisporus* in close proximity; an effect not observed when the inocula were separated at distance (Table 3.19).

3.1.2(v) The effect of an established biomass of T. harzianum in compost spatially separated from A. bisporus

The comparison of several membrane materials revealed that not all remained intact for the duration of the experiment suggesting that contact could occur between *T. harzianum* and *A. bisporus*. Nitrocellulose with pore size 0.1 μm and the Hybond membranes remained intact. Hybond-N was chosen for further studies.

The previous investigation involved the inoculation of *T. harzianum* as a relatively small number of spores. Therefore a similar experiment was set up where *A. bisporus* was spatially separated from a large established biomass of *T. harzianum* (produced in single tubes as described in 3.1.2(ii)). *T. harzianum* strain T7 (Th2) was used for this study.

An extensive, established mycelium of *T. harzianum* had no apparent effect on the growth of *A. bisporus,* which proceeded to colonise fully the compost on the other side of the membrane (data not shown - see Appendix).

3.1.2(vi) Saprophytic ability of T. harzianum in compost in the absence of A. bisporus

All strains of *T. harzianum* exhibited an ability, if somewhat limited, to colonise compost in the absence of *A. bisporus* providing an initial source of nutrients was present. The aggressive strains of *T. harzianum* displayed greater colonisation compared to non-aggressive strains. However, these observations were made from the controls of the previous experiments and therefore experiments specifically designed to investigate

a possible saprophytic mode of growth by *T. harzianum* were set up. *T. harzianum* spores were introduced to mushroom compost on 1) three sterilised rye grains, 2) three sterilised rye grains with a single grain of spawn added to the top of the tube and 3) three viable spawn grains. *T. harzianum* inoculum was added to the bottom of the tube. Tubes were filled with hand-compressed compost and the lids tightened and then released one quarter of a turn to provide aeration. Tubes were incubated at 25°C with high relative humidity (*ca.* 80%), in the absence of light.

Inoculation on rye grain (treatment 1) in the absence of *A. bisporus* revealed some genotypic differences between strains of *T. harzianum* and their ability to colonise mushroom compost (Table 3.20). The aggressive genotypes (2 and 4) of *T. harzianum* (strains T7 and RM10c) exhibited significantly higher colonisation than non-aggressive genotypes (1 and 3). Typically, aggressive strains exhibited growth 7.14-fold and 2.23-fold higher than Th1(c) and TD7 respectively (treatment 1). Strain TD7 (Th3) a non-aggressive strain, also exhibited substantial colonisation of compost. When a single spawn grain was added (treatment 2), strain TD7 appeared to be stimulated to a level similar to that of strain RM10c (Th4). The aggressive strains exhibited slightly less growth with treatment 2 than for treatment 1 (Table 3.20). Inoculation on spawn grain (treatment 3) appeared to stimulate the growth of strain T7, to a level very similar to that in treatment 1. Non-aggressive strains (Th1(c) and TD7) were reduced in growth compared to the other treatments (Table 3.20). All aggressive strains exhibited significantly higher growth than non-aggressive non-aggressive genotypes for treatments 1 and 3.

In short, aggressive strains (T7 and RM10c) produced significantly higher growth than that of non-aggressive strain Th1(c) for all treatments with the exception of T7 in treatment 2 (Table 3.20). Genotype 3 strain TD7 also displayed more substantial growth than strain Th1(c) for all treatments. Genotype 3 growth was less than that of aggressive strains (T7 and RM10c) for all treatments (except T7 in treatment 2), but this difference was not significant.

Table 3.20. Saprophytic growth of *T. harzianum* in compost.

Treatment*	Growth of *T. harzianum*** (mean ± SEM)			
	Th1(c)	TD7	T7	RM10c
1	6.3 ± 0.804^{a}	20.2 ± 5.705^{ab}	45.0 ± 5.00^{b}	45.0 ± 5.00^{b}
2	6.4 ± 1.204^{c}	43.9 ± 3.883^{d}	32.4 ± 6.539^{cd}	44.3 ± 4.971^{d}
3	4.5 ± 0.847^{e}	19.9 ± 5.201^{ef}	45.0 ± 5.00^{f}	31.3 ± 6.59^{f}

* Treatment 1: *T. harzianum* spores inoculated on three sterile rye grains; treatment 2: *T. harzianum* inoculated on three sterile rye grains with a single spawn grain added to top of tube; treatment 3: *T. harzianum* inoculated on three spawn grains.

** Growth assessed as on Table 3.8 legend.

Values followed by different letters (in rows) are significantly different according to the Friedman test for non-parametric data ($P<0.05$). *T. harzianum* strains: Th1(c) (G1), TD7 (G3), T7 (Th2) and RM10c (G4).

3.1.3 Microscopic studies of possible interactions between T. harzianum and A. bisporus

Dual cultures of *T. harzianum* and *A. bisporus* were examined by cryo-scanning electron microscopy. This technique was used to investigate the occurrence of mycoparasitic-like behaviour or structures produced by *T. harzianum* genotypes towards *A. bisporus* in a range of conditions. Earlier microscopic examinations were attempted with light microscopy, however the magnifications used failed to provide adequate detail as the hyphae of the two fungi were similar in size. Staining techniques using fluorescently tagged lectins were not suitable because *T. harzianum* and *A. bisporus* both have chitin-glucan cell walls and various lectins used corresponding to the haptens of these polymers failed to differentiate them. The increased depth of field and high magnifications possible with scanning electron microscopy revealed the presence of abundant crystals which were rich in calcium (determined by x-ray microanalysis using an energy disperser system) on the external surface of *A. bisporus* hyphae, which could be used as a defining feature. Dual cultures under various conditions were examined, including cultures on nutrient agar, growth in compost and *T. harzianum* on spawn grain.

3.1.3(i) Microscopic investigations of possible interactions between T. harzianum and A. bisporus on various nutrient agars

Agaricus bisporus and *T. harzianum* were inoculated on sterile cellophane discs on distilled water agar (DWA), malt extract agar (MEA) or filtered compost agar (fCA) [as described in section 3.1.1(i)]. Samples of the interaction zone (point of contact of the advancing edges of the two colonies) were removed from the agar on the cellophane for observation.

Generally, *T. harzianum* hyphae grew adpressed to the surface of the cellophane and *A. bisporus*, whilst growing on the surface, exhibited more aerial hyphae than *T. harzianum* (Fig. 3.3f). All *T. harzianum* genotypes exhibited self-coiling, perhaps indicative of self-parasitism, regardless of the nutrient agar medium. Evidence of mycoparasitism-like activity towards *A. bisporus* was provided by *T. harzianum* strain T7 (aggressive) on DWA and fCA but not on MEA (Fig 3.3). Behaviour characteristic of a mycoparasite included parallel growth along "host" hyphae, the production of hooked hyphal branches by the "mycoparasite" and hyphal branches that surrounded the "host" (Fig 3.3a-e). *T. harzianum* appeared not to coil around *A. bisporus* hyphae in the

manner observed in other systems (Benhamou and Chet 1993, 1996, 1997). The occurrence of such mycoparasitism-like behaviour over several samples from taken the interaction zones was relatively infrequent.

3.1.3(ii) Microscopic investigations of possible interactions between T. harzianum and A. bisporus within mushroom compost

Microscopic studies of interactions on nutrient agar provided an example of possible outcomes when *T. harzianum* and *A. bisporus* occupied the same space, however these *in vitro* experiments provide an artificial set of conditions. In an attempt to simulate the environment in which green mould infestations have occurred, tubes were inoculated with Th2, strains T7 and Th2A (spawn grain rolled in *T. harzianum* spores) and *A. bisporus* (spawn grain) at opposite ends in compost (compressed by hand). The tubes were incubated at 25°C, in the absence of light with high relative humidity (*ca.* 80%) for 3 weeks. Subsequently, samples of compost from interaction zones (point where the two colonies met) were removed and prepared for cryo-scanning electron microscopy.

Substantial growth was exhibited by both strains of *T. harzianum* and *A. bisporus*, however little *A. bisporus* growth was evident at the interface. Hyphal growth of *T. harzianum* strain T7 appeared to be along the surface of the compost and frequently appeared to adhere tightly to the compost (Fig 3.4a). *A. bisporus* exhibited more aerial growth than *T. harzianum* (Fig 3.4a). The detection of swollen *T. harzianum* hyphae in contact with *A. bisporus* hyphae may be indicative of mycoparasitism-like behaviour however, these observations were too infrequent to confirm this possibility (Fig 3.4b). Paradoxically there was also evidence of *A. bisporus* exhibiting mycoparasitism-like behaviour, albeit infrequently, towards *T. harzianum*, as demonstrated by the production of a hooked-branch by *A. bisporus* surrounding *T. harzianum* and the apparent penetration hole in a hypha of *T. harzianum* or *A. bisporus* (Fig 3.4c-d).

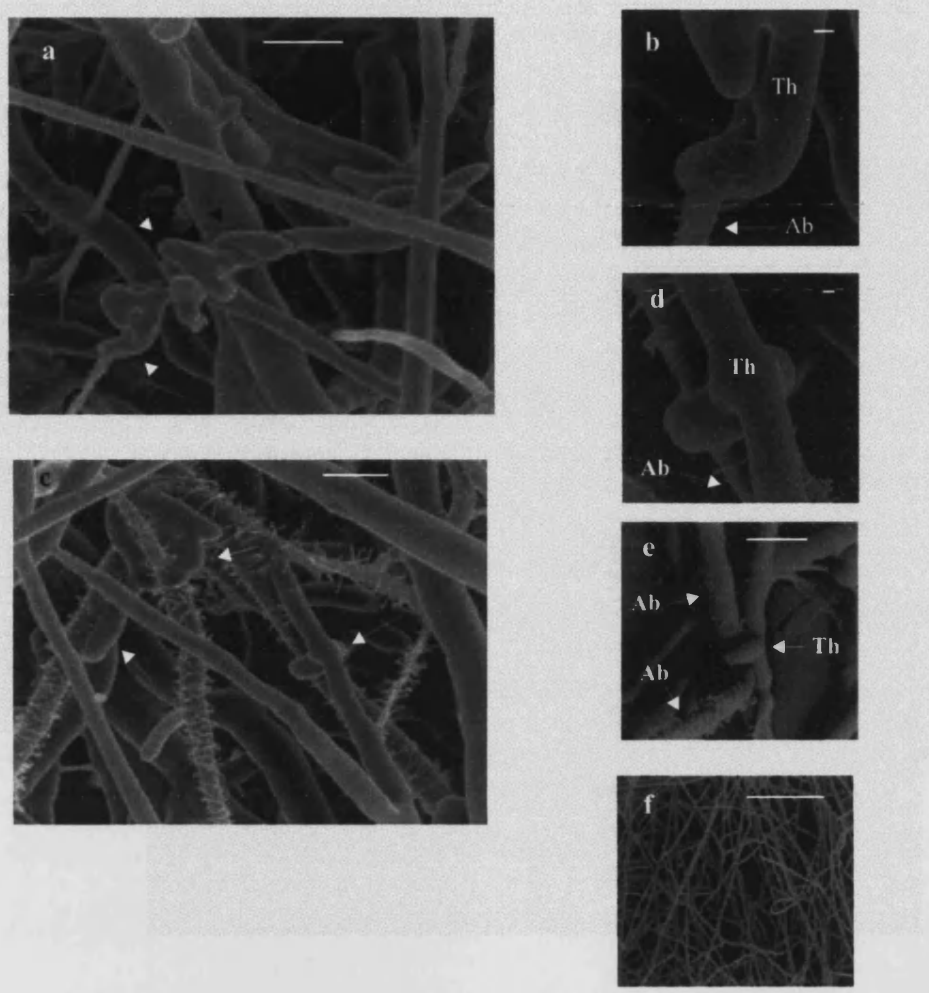


Figure 3.3: Cryo-scanning electron micrographs of interactions between *A. bisporus* and *T. harzianum* isolate T7 (Th2) on various nutrient media. *T. harzianum* hyphae have a smooth appearance and *A. bisporus* hyphae are coated in calcium-rich crystals. (a) *T. harzianum* produced several hooked-branches (arrows) around an *A. bisporus* hypha on filtered compost agar (bar = 10 μm); (b) enlarged area of (a) (bar = 10 μm). (c) mycoparasitism-like behaviour of *T. harzianum* on filtered compost agar towards *A. bisporus* exhibited as parallel growth (arrows) and hooked-branches (arrow) (bar = 1 μm); (d) an enlarged area of (c) (bar = 1 μm). (e) A hooked-branch around *A. bisporus* hypha, possibly produced by *T. harzianum* on distilled water agar (bar = 10 μm). (f) *A. bisporus* and *T. harzianum* on malt agar showing no apparent interaction (bar = 100 μm).

3.1.3(iii) Microscopic investigations of possible interactions between T. harzianum and A. bisporus on the inoculum base

Previous microscopic investigations considered the interactions that may occur when the advancing fronts of two colonies met having first established in separate areas. In many of the models of *T. harzianum* and *A. bisporus* in compost, the former was inoculated on *A. bisporus* spawn grain. The possibility that antagonism could occur at an earlier stage between live *A. bisporus* mycelium on the spawn grain and the *T. harzianum* was investigated by cryo-scanning electron microscopy.

Agaricus bisporus hyphal growth was induced from spawn grain on malt extract agar (MEA), incubated at 25°C in the absence of light. After 48 hours, hyphal growth was visible and individual spawn grains were coated in *T. harzianum* spores at one end only and returned to the 25°C incubator on MEA.

Hyphal growth of *A. bisporus* was apparent and in its presence germination of *T. harzianum* spores and hyphal growth occurred (Fig 3.5a). There was more aerial hyphal growth produced by *A. bisporus* than by *T. harzianum* (Fig 3.5). Fungal growth appeared to be independent and mycoparasitism-like behaviour was evident only very infrequently. In one instance *T. harzianum* strain T7 produced a hyphal swelling when in close contact with a hypha of *A. bisporus* (Fig 3.5c). One incident of *T. harzianum* coiling around *A. bisporus* was recorded; the "mycoparasite" also grew along the *A. bisporus* hypha and appeared to prevent production of or degrade the crystals rich in calcium on the hypha (Fig 3.5b).

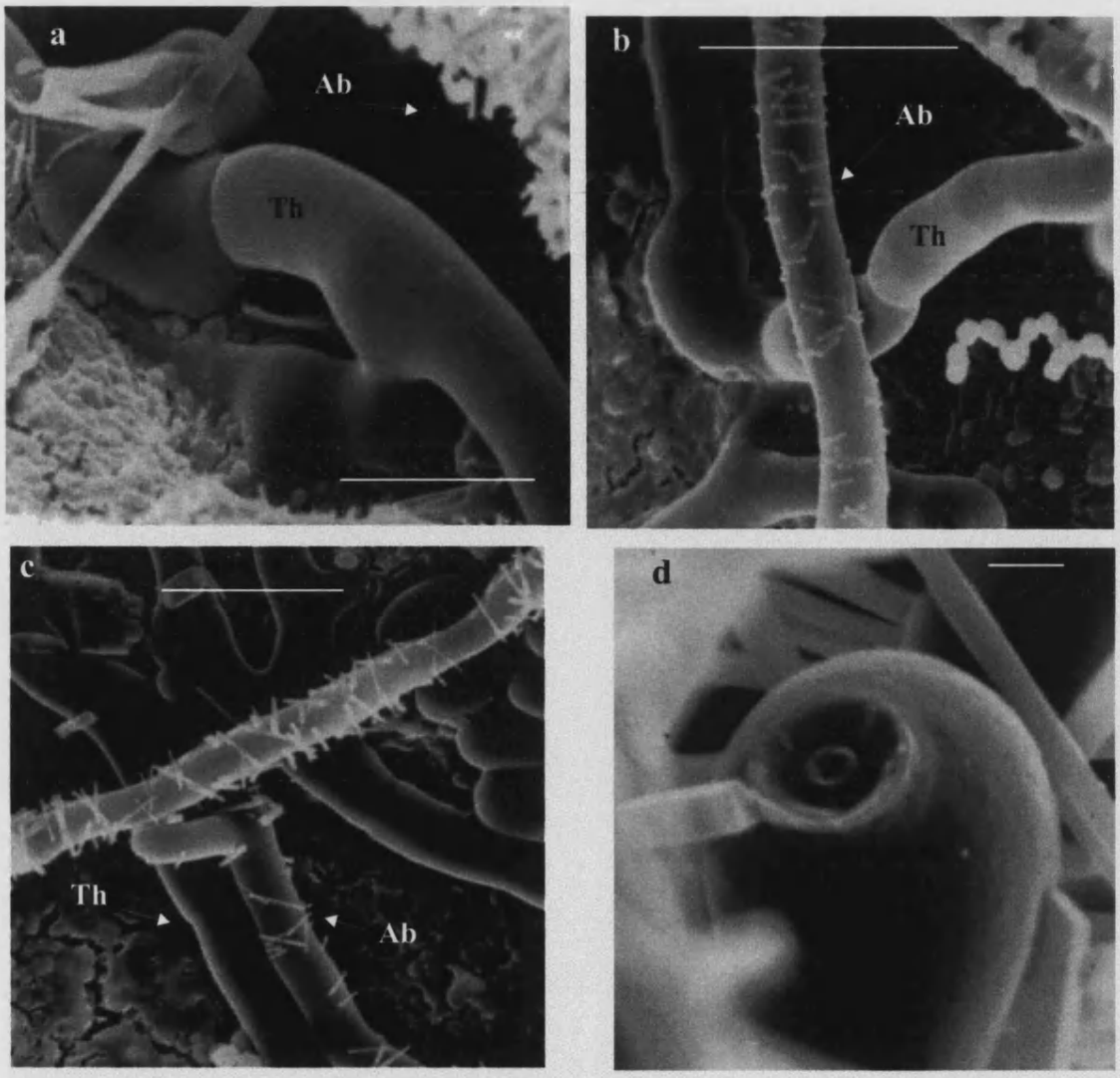


Figure 3.4: Cryo-scanning electron micrographs of interactions between *A. bisporus* and *T. harzianum* (isolate T7) in non-sterile compost. (a) *A. bisporus* and *T. harzianum* on surface of straw with no apparent interaction. *T. harzianum* appears to be tightly adpressed to straw surface. (b) Hyphal swelling of *T. harzianum* in close proximity of *A. bisporus* hypha. (c) Hooked-branch produced by *A. bisporus* around *T. harzianum*. (d) A hypha that appears to have been penetrated (and the invading fungus subsequently removed). Bars represent 10 μm (except in (d) where bar indicates 1 μm).

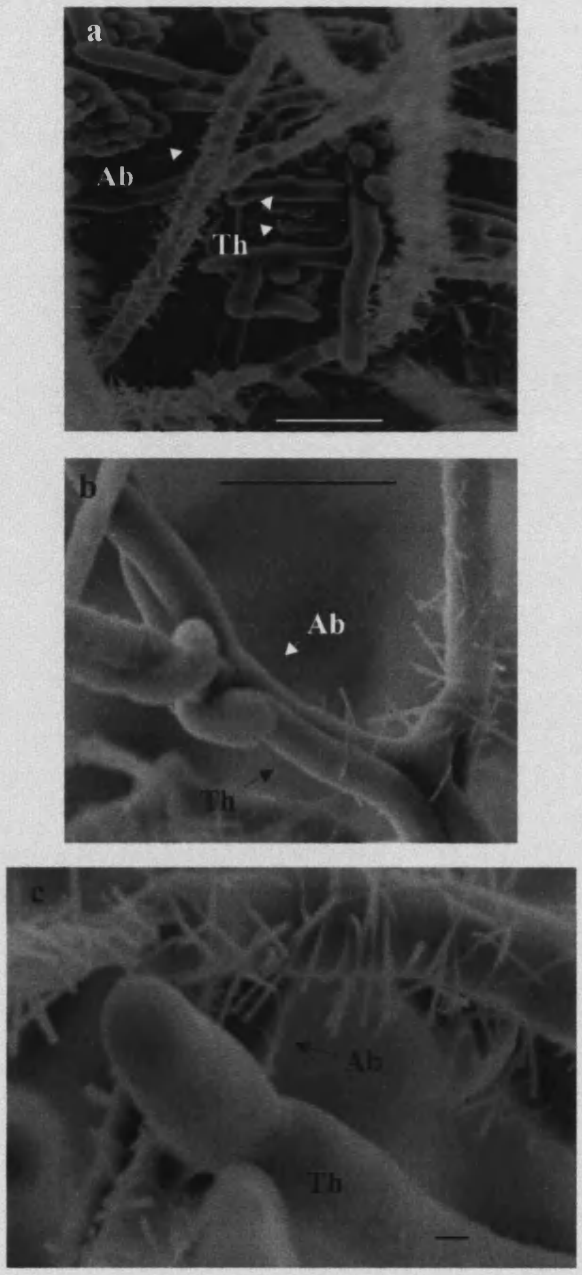


Figure 3.5: Cryo-scanning electron micrographs of interactions between *T. harzianum* (isolate T7) and *A. bisporus* on spawn grain. (a) Germinating spores of *T. harzianum* and aerial hyphae of *A. bisporus* with no apparent interaction. (b) Coiling of *T. harzianum* around *A. bisporus* or self. (c) Hyphal swelling of *T. harzianum* in contact with *A. bisporus*. Bars represent 10 μm (except (c) where bar indicates 1 μm).

3.2 Discussion

3.2.1 Interactions between T. harzianum and A. bisporus in in vitro cultures

In vitro plate assays provided limited explanations of possible antagonism between *T. harzianum* and *A. bisporus* since only a restricted set of conditions could be provided. Within mushroom compost possible interactions between *T. harzianum* and *A. bisporus* would rarely be as simplistic due to the presence of an extensive microflora and changing environmental conditions. The clear limitation of the plate assays was reduced by using a range of nutrient media that differed in nutrient concentrations and solubility thus providing nutrients of varying availability or accessibility.

Competition between *T. harzianum* and *A. bisporus* was revealed since mutual inhibition was exhibited on MEA and DWA. This suggests that although nutrients were limiting, both fungi were able to obtain sufficient nutrients for growth, albeit at reduced rates compared to the controls. Competition may continue until nutrients are depleted; at which point the balance may alter in favour of the dominant fungus or there may be a change in strategy of antagonism. Alternatively, the mutual inhibition could be due to the production of diffusible antibiotics by both fungi (Dennis and Webster 1971c).

On fCA *A. bisporus* was dominant initially, exhibiting stimulated growth while Th1 and Th2 were apparently unaffected (Table 3.1). Eventually mutual inhibition was observed indicative of competition. Therefore although *A. bisporus* was better adapted than *T. harzianum* to colonise fCA, *T. harzianum* was able to compete as nutrients became limited perhaps due to derepression of depolymerases (Ilmen *et al.* 1996).

A. bisporus growth was inhibited on MEA by non-volatile, putative antibiotic compounds produced by Th1 and Th2 and this supports previous reports (Mumpuni *et al.* 1998). However, in contrast to this work, Mumpuni *et al.* (1998) found that Th2 exhibited less inhibitory effects compared to inhibition in the presence of Th1 and Th3. Inhibition of *A. bisporus* was not the result of nutrient depletion following previous *T. harzianum* growth since Th2, but not Th1, inhibited *A. bisporus* on DWA and fCA. Different growth effects associated with the various nutrient media may indicate dissimilar modes of antagonism according to nutrient availability. Th1 may only produce non-volatile antibiotics when nutrients are in excess and when limited, revert to

survival tactics of self-propagation (Pianka 1970). *T. harzianum* genotypes exhibited highest inhibition of *A. bisporus* on MEA and similarly Whipps (1987a) reported the highest *T. harzianum* non-volatile antibiotic activity towards a range of fungi on a high nutrient agar.

Trichoderma harzianum genotypes produced volatile compounds with varying effects on *A. bisporus*, according to the medium used. Th2 volatile substances stimulated *A. bisporus* on all media except DWA and Th1 volatile compounds stimulated *A. bisporus* on MEA and fCA. In contrast, previous work suggested that volatile compounds of Th1, Th2 and Th3 were inhibitory towards *A. bisporus* on MEA (Mumpuni *et al.* 1998). Th2 was more tolerant of and in some cases stimulated by *A. bisporus* volatiles compared to the non-aggressive genotype and this difference was also reported by Mumpuni *et al.* (1998) following the effect of metabolites produced by *A. bisporus* in liquid cultures.

Paradoxically, *A. bisporus* and Th2 stimulated one another on fCA and it is suggested here that Th2 may support the *A. bisporus* mycelium (and under certain conditions, *A. bisporus* may support Th2) until a change in environmental conditions triggers antagonism. Similarly, Mumpuni *et al.* (1998) argued that stimulation of Th2 by *A. bisporus* metabolites ensures colonisation by both fungi until growth is maximal and the competitive balance then changes in favour of Th2, which subsequently inhibits fructification.

The effects of putative non-volatile and volatile antibiotics may also alter according to the water potential of the medium since antagonists had little effect on pathogens on media with high water potentials (Whipps and Magan 1987). The optimal water content of mushroom compost is 69% at spawning and this falls during mycelial growth (Gerrits 1988), therefore antibiosis may be more important after colonisation.

Temperature fluctuations may change the effects of antibiotic compounds and the outcomes of competitive interactions (Schoeman *et al.* 1996; Fletcher 1997). The inhibitory effects of *T. harzianum* diffusible metabolites on interactions with basidiomycete isolates were more acute at 10°C than at 22°C according to Schoeman *et al.* (1996). Fletcher (1997) found that *T. harzianum* established mycelial growth at a faster rate than *A. bisporus* at low temperatures and was therefore capable of colonising

the spawn grain before *A. bisporus* had established mycelium. Addition of Th2 had no effect on compost fully colonised by *A. bisporus* and therefore early growth stages of *A. bisporus* may be particularly vulnerable to antagonists (Fletcher 1997). Perhaps with a different temperature regime in addition to different nutrient media, the effects of *T. harzianum* and *A. bisporus* antibiotic compounds may be more pronounced.

3.2.2 Interactions between T. harzianum and A. bisporus in a model of the commercial situation of T. harzianum green mould

Conditions with a closer analogy to the commercial situation were achieved by inoculating tubes containing mushroom compost with *T. harzianum* and *A. bisporus*.

An assessment of various inoculum bases revealed a nutrient base as prerequisite for colonisation of compost by *T. harzianum*. The addition of Th2 (T7) as a spore suspension had no effect on the growth of *A. bisporus*. Fletcher (1997) reported a similar requirement when *T. harzianum* spores were introduced on vermiculite (an inert material with no nutritional content) since reduction of *A. bisporus* growth only occurred when inoculum was adjacent to spawn. Previously *T. harzianum* was suggested to be an obligate commensal/parasite and its host was *A. bisporus* (Seaby 1987). *T. harzianum* may instead, require an initial source of nutrients in order to colonise mushroom compost which would, in the commercial situation, be provided by the grain of mushroom spawn.

Th1 and Th2 established growth from a rye grain inoculum base in the absence and presence of *A. bisporus* in compost, thus confirming the requirement of a nutrient source. Adjacent to spawn Th2 caused *ca.* 67 to 100% inhibition of *A. bisporus* growth, which was reduced to *ca.* 13% when the inocula were separated; an effect also reported by Fletcher (1997). Therefore the establishment of a dense mycelium by *A. bisporus* may diminish the effects of Th2 and this supports observations made in the field (Seaby 1996b). The growth of Th2 adjacent to the spawn did not differ from the controls with *T. harzianum* alone, reiterating the importance of a nutrient source. Furthermore, the rapid and efficient assimilation of the nutrient source appeared to be vital to enable *T. harzianum* colonisation before the establishment of *A. bisporus*

Th1 exhibited substantially less growth than other genotypes and there was little difference in the presence or absence of *A. bisporus*. This limited colonisation also required a nutrient source and had no effect on *A. bisporus* growth. Also Sharma *et al.* (1999) found that Th1 and Th3 colonised phase II compost up to 50% of the level determined for Th2, with no adverse effects on *A. bisporus*.

The outcome of inoculating *T. harzianum* on spawn was of interest since spawn represented an obvious route of infection or contamination of compost (Staunton 1987; Seaby 1989; 1996b). *T. harzianum* genotypes on spawn inoculum exhibited inhibition of *A. bisporus* growth from spawn, but only Th2 caused significant reduction in growth. *T. harzianum* growth from spawn grain was similar in the presence and absence of *A. bisporus* and Th2 exhibited greater colonisation than other genotypes. The reduction in growth of *A. bisporus* by Th2 supported previous reports (Seaby 1996b). This suggests that Th2 is an efficient assimilator of available nutrients and is tolerant of *A. bisporus*.

Colonisation of compost by *T. harzianum* from inoculated on grain bases suggests a requirement for a nutrient base rather than a requirement for *A. bisporus* mycelium as a source of nutrition. Nevertheless this does not dismiss the possibility of dependency on *A. bisporus* for continued growth by *T. harzianum* under different conditions or at a later stage in colonisation.

Since aggressive *T. harzianum* strains were rarely isolated from compost raw materials (Seaby 1987) and contaminated spawn was a plausible source of infection, *T. harzianum* infested spawn was used in further tests. The effects of *T. harzianum* genotypes from spawn inoculum on *A. bisporus* growth verified the inhibitory effects of Th2. Inoculation of *T. harzianum* on "viable" and "non-viable" spawn grain (*A. bisporus* killed by heat treatment) revealed some inhibitory effects of viable spawn grain on *T. harzianum* growth. Viable spawn grain held a higher spore load than non-viable, however higher spore loads did not result in greater growth. Also a strain with a high spore load (Th1(c)) and a strain with a low spore load (Th2F) both exhibited low levels of growth.

Higher growth of *T. harzianum* was achieved from non-viable spawn than that from the viable spawn (Table 3.17), indicating that the presence of live *A. bisporus* mycelium on

the spawn slightly reduced the growth of *T. harzianum*. Non-viable spawn may present a more readily available source of nutrients. However, to attempt a realistic model of possible infection routes into the commercial situation, the use of viable spawn grain inoculum carrier was continued.

T. harzianum failed to produce inhibitory volatile compounds in response to *A. bisporus* in compost and similar observations were made for *T. harzianum* against *R. solani* (Dennis and Webster 1971b). However, the plate assays earlier in this work detected stimulation of *A. bisporus* by aggressive strains of *T. harzianum*; likewise Dennis and Webster (1971b) found that the growth of *R. solani* in the presence of *T. harzianum* was slightly stimulated at certain sample times (Dennis and Webster 1971b). Use of the compost model did not reveal any stimulation in the growth of *A. bisporus* but this would only have been apparent if assessments of *A. bisporus* growth had been compared at various time points. Sampling time may be very important as only ageing *T. harzianum* cultures produced two butenolide compounds and this coincided with a reduction in the proportion of fatty acids and glyceride (reviewed by Ghisalberti and Sivasithamparam 1991).

This work contradicts reports that *T. harzianum* volatiles were inhibitory to *A. bisporus* (Mumpuni *et al.* 1998), rather it supports the idea that the fungi may benefit from the presence of each other initially until the competitive balance changes at the expense of one of them (Mumpuni *et al.* 1998). Furthermore, *T. harzianum* inhibitory volatiles produced in the presence of *R. solani* and wheat straw, had a characteristic coconut aroma and were reported to be alkyl pyrones (Claydon *et al.* 1987). All the isolates of *T. harzianum* used in this study produced a coconut aroma, particularly non-aggressive Th3, on nutrient agar. In compost *T. harzianum* may have produced alkyl pyrones to which *A. bisporus* was tolerant. However, Claydon *et al.* (1987) also reported that the high concentrations of volatiles *in vitro* may not be matched *in vivo* (Claydon *et al.* 1987).

Inhibition of *T. harzianum* (T7) by *A. bisporus* in compost when inocula were separated physically but positioned close to the membrane is suggestive of the production of short-range volatile antibiotics by *A. bisporus*. The effect was not observed when inocula were separated by 100 mm. An established *T. harzianum* mycelium may be

more tolerant of such compounds than germinating spores in the vicinity of *A. bisporus*. Inhibition of *T. harzianum* (T7) by apparent short range volatiles of *A. bisporus* was also observed in this work on bCA, a sterile agar medium with some analogy to compost with respect to insoluble carbon sources. *A. bisporus* volatile substances in compost may have different effects on other *T. harzianum* genotypes.

A large established mycelium of *T. harzianum* (T7) had no inhibitory effects on *A. bisporus* growth when separated by a gas permeable membrane. Therefore the production of critical levels of volatile antifungal compounds by *T. harzianum* appeared not to be dependent on biomass of the antagonist.

Trichoderma harzianum genotypes colonised the compost from rye grain in the absence of *A. bisporus* and this is thought to be the first report of the saprophytic growth capabilities of *T. harzianum* genotypes associated with mushroom compost. Th2 and Th4 displayed substantial colonisation of compost compared to the limited growth of Th1, indicating the evolution of survival strategy by Th2 and Th4. Th3 displayed less growth than the aggressive genotypes (2 and 4) but more than Th1. Since Seaby (1996b) reported that Th2 conidia were inhibited on cultures of bacteria isolated from compost, it is unlikely that greater saprophytic growth demonstrated by Th2 and Th4 could be due to a tolerance of the microflora. The varying saprophytic growth capabilities of *T. harzianum* genotypes may suggest differences in the carbon metabolism and the depolymerases produced to obtain necessary nutrients. Generally Th1 colonisation displayed strategies associated with ruderal fungi, for example the production of vast numbers of reproductive propagules (Pianka 1970). In contrast, the aggressive genotypes were more combative, illustrated as delayed sporulation and rapid mycelial colonisation. Th3 exhibited behaviour of both ruderal and combative fungi since the extent of colonisation was at a similar level to that of aggressive strains, but sporulation occurred earlier than the aggressive strains.

The presence of one spawn grain at a distance of 50 mm or three spawn grains adjacent to *T. harzianum* inoculum had no effect on the saprophytic growth of Th2, Th3 or Th4 from rye grain. The growth of these *T. harzianum* genotypes was identical in the presence and absence of *A. bisporus*, which therefore confirms that *T. harzianum* is not

dependent on *A. bisporus* as a host. Th1 exhibited reduced growth when spawn grain was adjacent, which indicates less tolerance to *A. bisporus* than by the other genotypes.

3.2.3 Ultrastructural observations of possible interactions between T. harzianum and A. bisporus

Microscopic observation of interactions between *T. harzianum* and *A. bisporus* on various substrates failed to confirm mycoparasitism. Examples of mycoparasitism-like behaviour by *T. harzianum* were infrequent and thus indicated a competitive relationship between the two fungi. Fletcher (1997) also proposed that *T. harzianum* was a competitor because of lack of evidence for pathogenicity. However *T. harzianum*, a ruderal fungus but with genotypes capable of combative behaviour to colonise compost, may be able to change strategies. Therefore it is possible that Th2 is capable of mycoparasitism but only under conditions of nutrient stress and until that point, Th2 may function as a competitor.

Examples of mycoparasitism from interaction zones only occurred on low nutrient agar, which may be indicative of stress-induced behaviour. *T. harzianum*, is innately r-selected in growth characteristics and may respond to nutrient stress before slow growing *A. bisporus* which is K-selected (Pianka 1970). Cell wall degrading enzymes of the plant pathogens *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* were regulated by derepression, such as exposure to tomato stem cell walls (Cooper and Wood 1975). Perhaps it is the difference in the competitive strategies of *T. harzianum* and *A. bisporus* that determines the outcome of the interaction.

The existence of *T. harzianum* as a competitor in mushroom compost was also evident from microscopic observations of interactions in compost. The attachment to and parallel growth of *T. harzianum* mycelium on the wheat straw component of compost suggested that nutrients were obtained from enzymatic degradation of the available cellulosic and hemicellulosic resources. Since some characteristic features of antagonism such as appressoria are thought to be induced by derepression (Emmett and Parbery 1975) and there was little evidence of contact or antagonism between *T. harzianum* and *A. bisporus* it would appear that neither fungus was exposed to conditions of nutrient stress.

The calcium-rich crystals on the external surface of the *A. bisporus* mycelium detected by SEM EDX-ray microanalysis were probably calcium oxalate since *A. bisporus* has been reported to sequester excess calcium in this manner (Atkey and Wood 1983; Dutton *et al.* 1993). Masaphy *et al.* (1987) suggested these crystalline structures may prevent the formation of *A. bisporus* reproductive strands; analogously and critically the crystals may prevent contact with *T. harzianum*. This would represent a novel form of 'defense' for a microorganism.

In many *T. harzianum* systems involving various phytopathogens the roles of mycoparasite and host respectively have been clearly evident (Elad *et al.* 1980, 1983a). *T. harzianum* is known to parasitise many plant pathogens including *R. solani* and *S. rolfsii* (Elad *et al.* 1980), *P. aphanidermatum* (Sivan *et al.* 1984), *F. oxysporum* (Sivan and Chet 1987) and *B. cinerea* (Lorito *et al.* 1996) and hence there are many reports on the potential of *T. harzianum* as a biocontrol agent (Papavizas 1985; Chet 1987). In general, the mechanism of mycoparasitism is common in systems of antagonism involving *T. harzianum*. The evidence for mycoparasitism includes penetration structures to enable the invasion of the host by *T. harzianum* (Elad *et al.* 1983a) and cytochemical studies have revealed the degradation of host cell walls by hydrolytic enzymes of *T. harzianum* (Benhamou and Chet 1993, 1996).

In this work evidence points to a different mode(s) of antagonism. The establishment of both *T. harzianum* and *A. bisporus* appears to be dependent on the following: a) the genotype of *T. harzianum*, b) position of inocula, c) the extent of the established *A. bisporus* mycelium, d) presence of a grain nutrient base for *T. harzianum* and e) physical contact between fungi.

The nutrient uptake mechanisms of *T. harzianum* genotypes may be of importance since Altomare *et al.* (1999) reported the ability of *T. harzianum* to solubilize phosphates and other micronutrients by chelation and redox mechanisms. In competitive interactions Th2 and Th4 may also be capable of chelating such nutrients from compost thus depriving *A. bisporus* of essential micronutrients. The rapid growth rate of Th2 and Th4 in compost may be indicative of nutrient uptake systems, which could be far more efficient than those of *A. bisporus*.

T. harzianum genotypes demonstrated differences in their tolerance of *A. bisporus* putative inhibitory metabolites; the aggressive genotypes exhibited higher tolerance than non-aggressive strains in competitive situations and to volatile compounds of *A. bisporus* origin.

The enhanced saprophytic growth ability of the aggressive *T. harzianum* genotypes was the most significant discovery of this section of research and was the first obvious distinction from Th1. This supports the theory that *T. harzianum* is a competitor within the compost environment. It is proposed that compost at spawning stage is particularly vulnerable to contamination with aggressive *T. harzianum* since *T. harzianum* may utilise the spawn grain as a nutrient source. The 'exploratory' growth employed by Th2 and Th4 with delayed sporulation may be sufficient and rapid enough to allow saprophytic growth between the spawn grains. This early *T. harzianum* infestation may then outcompete *A. bisporus*.

In summary the research here and the available literature indicates a competitive interaction dependent on the availability of a nutrient source for saprophytic growth of *T. harzianum* and the time at which contamination occurs. The extent of the respective biomasses of *T. harzianum* and *A. bisporus* also affects the outcome. Evidence suggests that mycoparasitism by *T. harzianum* is possible but is determined by conditions in which the mycelium is stressed.

Chapter Four

Agaricus bisporus cell wall-degrading enzymes from *Trichoderma harzianum*

4.0 Introduction

Trichoderma harzianum, a known mycoparasite of several phytopathogens, secretes many lytic enzymes corresponding to host cell wall components during antagonism (Elad *et al.* 1982). Chitin and glucan are the major structural polymers of group V fungi, which include *A. bisporus* (Bartnicki-Garcia 1968). The structure of *A.bisporus* cell walls consists of different layers of integrated polysaccharides and proteins. This structure may require the action of several enzymes working synergistically for complete degradation. A layer of chitin microfibrils is embedded in a β-glucan and protein matrix, this is shielded by a layer of alkali-soluble α-glucan (Michalenko *et al.* 1976). The external surface is coated in a β-glucan mucilage.

Development of *A.bisporus* coincides with changes in the chemical composition of the cell wall (Garcia Mendoza *et al.* 1987; Mol and Wessels 1990). The difference in cell wall structure between vegetative and reproductive mycelia is due to a switch in the mode of growth of the reproductive mycelia. Hyphae in expanding mushrooms enlarge by diffuse extension, this occurs over the complete cell wall surface as opposed to apical extension, which occurs in vegetative mycelium. This enhanced ability to extend over the whole wall surface is correlated to an increase in the proportion of (1,6)-β-linked glucose in side branches of β-glucan (Mol and Wessels 1990). Aggressive genotypes of *T.harzianum* appear to develop during the early stages of *A. bisporus* colonisation and therefore may encounter a relatively low proportion of (1,6)-β-linkages. The negative effect on mushroom development, provoked by aggressive *T. harzianum* occurs before fruiting bodies emerge (Seaby 1987).

The mycoparasitic action of *T. harzianum* towards several fungi with chitin-glucan walls has targeted research towards chitinases and glucanases (Carsolio *et al.* 1994; De la Cruz *et al.* 1995). Proteases have also been targeted, since they have been shown to play an important role in the parasitism of *R. solani* by *T. harzianum* (Geremia *et al.* 1993; Flores *et al.* 1997). Proteases could have bifunctionality, working in synergy with chitinases and

glucanases during the degradation of cell walls but may also degrade cell contents on invasion of the host (Geremia *et al.* 1993). The production of various polysaccharidases enables penetration of host cell walls and release of, or access to a source of nutrients. The complex nature of fungal cell walls is likely to require degradation by different depolymerases (Elad *et al.* 1983a); these may act simultaneously or sequentially and often several enzymes work in synergy (De la Cruz *et al.* 1992, 1995b).

Competent degradation of the fungal cell wall depends on tight regulation of depolymerase genes. A common trigger is thought to induce many enzymes secreted during mycoparasitism and several genes have been assigned to chromosome VI of *T. harzianum* (Herrera-Estrella *et al.* 1993; Carsolio *et al.* 1994). Some proteinases as well as chitinases are induced by chitin or chitin-containing substrates (Geremia *et al.* 1993). Depolymerases, in particular chitinases, may also be triggered during the early stages of mycoparasitism following recognition (Inbar and Chet 1995). Differential regulation is also evident since various chitinases exhibit induction by de-repression while others are only induced by the presence of chitin or chitin-containing materials (Limon *et al.* 1995). Many of these depolymerases are tightly controlled by catabolic repression in the presence of glucose or readily utilisable sugars (Lorito *et al.* 1996b). The carbon catabolite repressor protein (Cre1) has been implicated.

Isoforms of depolymerases may be the products of different genes or formed by post-translational modifications such as proteolysis or glycosylation (Goyal *et al.* 1991). Degradation of certain endoglucanases of *T. reesei* with α-mannosidase, resulted in products of size identical to possible EG precursors (Messner and Kubicek 1988). Several *T. harzianum* depolymerases have a recognition site for the proteinase Kex 2; the subtilisin-like proteinase Kex 2 processes secreted proteins in mammalian, yeast and fungal cells. Proteinase Prb1 (Geremia *et al.* 1993), glucanase BGN.16.2 (Lora *et al.* 1995) and endochitinase CHIT42 (Garcia *et al.* 1994) all contain a Kex 2 recognition site.

The aims of this chapter are to investigate the ability of *T. harzianum* to respond to cell walls of *A.bisporus* as a sole carbon source. Consideration was paid to different genotypes of *T.harzianum* and their capacity to secrete the necessary depolymerases. As well as

studying the total activity of various cell wall-degrading enzymes, this chapter describes a detailed investigation of isoenzymes produced by the four *T. harzianum* genotypes.

4.1 Results

4.1.1 Production of cell wall depolymerases by T. harzianum in the presence of A.bisporus cell walls

Trichoderma harzianum was cultured in liquid medium (Cooper and Wood 1975) supplemented with 1% w/v glucose. Mycelial dry weight was determined after three days incubation at 25°C; these conditions produced sufficient growth (*ca.* 500 mg dry weight) to enable transfer of mycelium to induction conditions, subsequent to 12 hours starvation (basal medium only). Mycelium was subjected to induction by the presence of *A. bisporus* cell walls and samples of culture fluids were removed after 12, 24, 48 and 72 hours for assay. Culture filtrates were tested for total chitinase, β-1,3-glucanase and protease activities per gram dry weight mycelium.

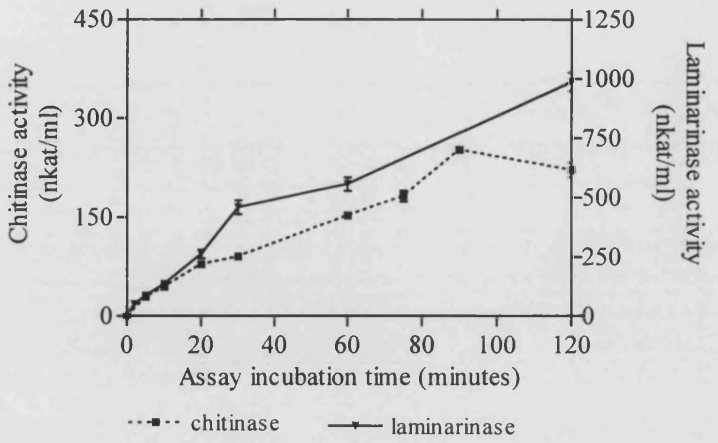
Partial characterisation of the enzyme classes was determined by performing enzyme assays under a range of conditions. Optimal pH, temperature and assay incubation times were determined. The pH range encompassed pH 5.0 to 7.0 in half unit steps using the buffer systems MES and MOPS. Temperature range spanned 30 to 60°C and incubation times were chosen in the range 2 to 120 minutes.

4.1.1(i) Optimum pH, temperature and incubation time for laminarinase activity

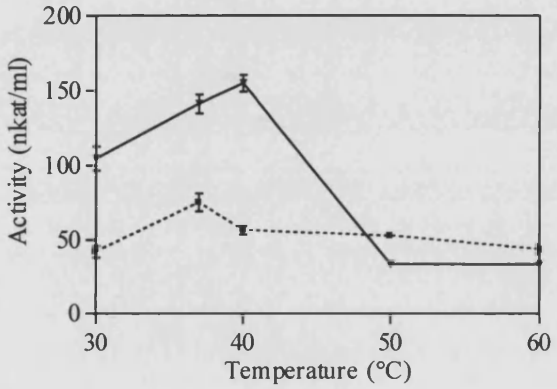
The increase in laminarinase activity was linear between 0 and 30 min. Beyond 30 rate of the reaction was reduced and non-linear (Fig 4.1a). An optimal incubation time of 30 min was determined.

Laminarinase activities increased between incubation temperatures of 30 to 40°C, reaching maximal activity at 40°C, beyond which there was rapid decrease in activity (Fig 4.1b). An optimal incubation temperature of 37°C was chosen in order to coincide with chitinase activity (see below) and enable a more efficient and manageable set of assay conditions.

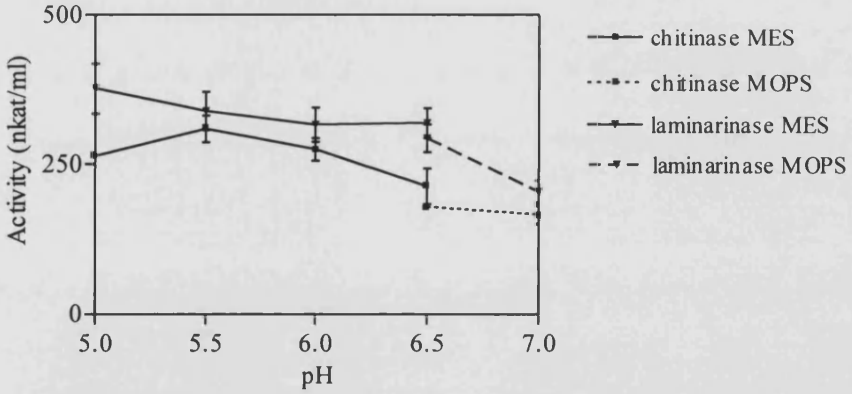
Laminarinase activity over pH range 5.0 to 6.5 exhibited little difference, however with pH in excess of 6.5 there was a substantial drop in activity (Fig 4.1c). In addition, the buffer appeared to have a slight effect, as activity in MOPS at pH 6.5 was lower than activity



a



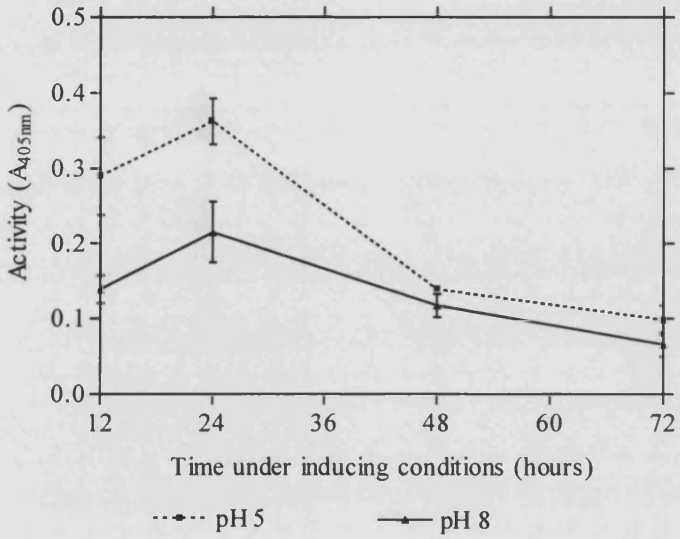
b



c

Figure 4.1: Optimal conditions of incubation time, temperature and pH for laminarinase and chitinase assays: a) optimum assay incubation time; b) optimum temperature; c) optimum pH. Bars indicate standard error of the mean. All activities calculated as nkat/ml/g dry weight of mycelium.

Figure 4.2: Comparison of two pH regimes to determine temporal production of protease activity.



Bars indicate standard error of the mean. All activities calculated as Abs_{405nm}/g dry weight.

in MES at pH 6.5. The highest laminarinase activity occurred at pH 5.0 buffered in MES (Fig 4.1c) and thus this was determined as optimal.

4.1.1(ii) Optimum pH, temperature and incubation time for chitinase activity

Chitinase activity exhibited a linear increase between incubation times of 0 to 60 minutes (Fig 4.1a). Incubation between 60 and 90 minutes exhibited a non-linear increase in activity. Therefore an optimal incubation time of 60 minutes was determined.

Chitinase activity was not markedly affected by temperature between 30 and 60°C, however maximal activity exhibited at 37°C was chosen as the optimum for assay (Fig 4.1b).

Chitinase activity was higher at the pH range buffered by MES in which activity peaked at pH 5.5 (Fig 4.1c). An optimum of pH 5.5 was resolved.

4.1.1(iii) Comparison of two pH regimes to determine temporal production of protease activity

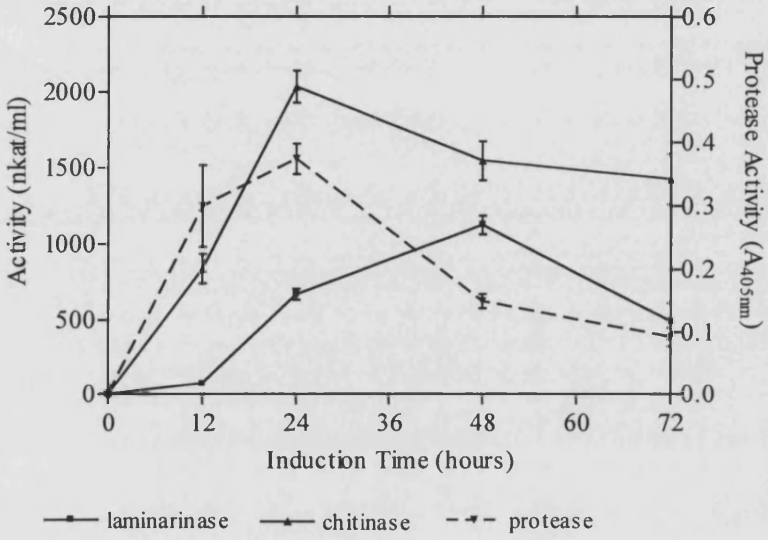
General protease activity was determined using the substrate azocasein. Overall protease activity during this assay was greatly affected by pH (Fig 4.2). Activity at pH 8.0 was substantially reduced compared to that at pH 5.0. Therefore, pH 5.0 was determined as optimal.

The determination of overall protease activity on the azocasein substrate was not as sensitive as other methods. Activity could only be determined after 3 hours incubation at 37°C.

4.1.1(iv) Sequential production of cell wall depolymerases by T.harzianum in the presence of A. bisporus cell walls

Sequential secretion of depolymerases in the presence of *A. bisporus* cell walls by *T. harzianum* strain T7 (G2) was investigated (Fig 4.3). Protease and chitinase activity increased rapidly in the first 12 hours of incubation and both continued to increase up to 24 hours incubation. After a 12 hour delay, laminarinase activity rapidly increased

Figure 4.3: Sequential production of cell wall degrading enzymes by *T. harzianum* with *Agaricus* cell walls as the sole carbon source.



Bars indicate standard error of the mean. All activities calculated as nkat/ml/g dry weight of mycelium.

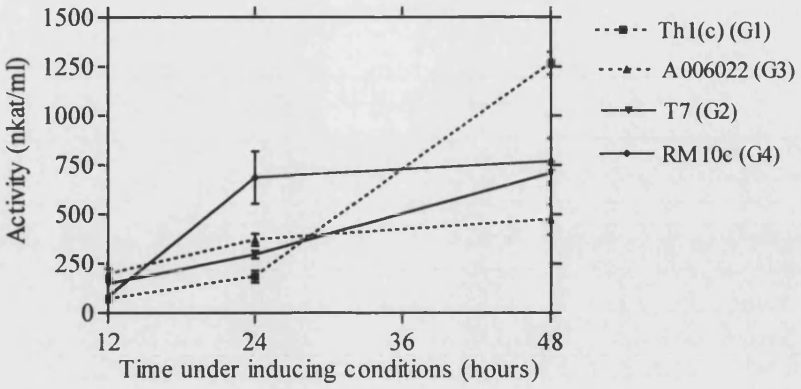


Figure 4.4: Comparison of temporal production of laminarinase by *T. harzianum* genotypes. *T. harzianum* strains: Th1(c) (Th1), A006022 (Th3), T7 (Th2) and RM10casing (Th4). Bars indicate standard error of the mean (SEM). Activity calculated as nkat/ml/g dry weight of mycelium.

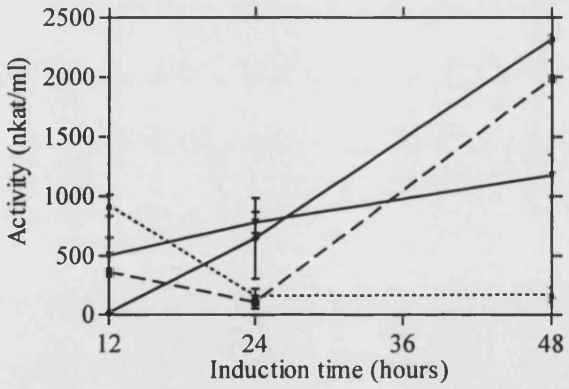
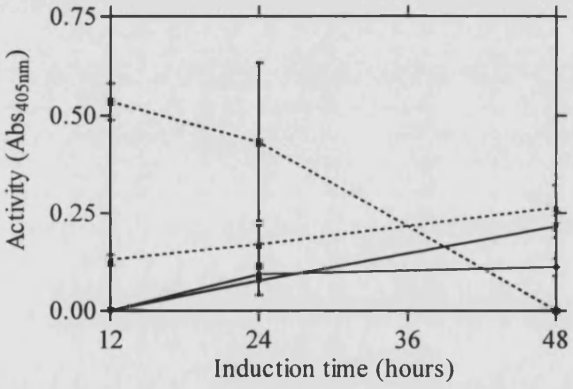


Figure 4.5: Comparison of temporal production of chitinase by *T. harzianum* genotypes. For description of *T. harzianum* strains see Fig. 4.4. Bars indicate SEM. Activity calculated as nkat/ml/g dry weight of mycelium.

Figure 4.6: Comparison of temporal production of protease by *T. harzianum* genotypes.



For description of *T. harzianum* strains see Fig 4.4. Bars indicate SEM. Activity calculated as nkat/ml/g dry weight mycelium.

between 12 and 24 hours of induction and continued to increase, reaching maximum activity after 48 hours under these inducing conditions.

4.1.2 Comparison of the production of depolymerases by T. harzianum genotypes

Temporal production of several depolymerases was compared between *T. harzianum* genotypes under conditions of induction, ie. in the presence of *A. bisporus* cell wall extract. This was to investigate the possibility of distinguishing strains according to total enzyme activity triggered under mycoparasitic-like conditions and to investigate the potential roles of the individual enzymes.

4.1.2(i) Comparison of temporal production of laminarinases by T.harzianum genotypes

Laminarinases (β-1,3-glucanase) were produced by all genotypes of *T. harzianum* (Fig 4.4). Activity of laminarinases of all genotypes increased in the first 24 hours under inducing conditions and subsequently activity of genotypes 2, 3 and 4 reached a plateau. Genotype 1 exhibited a sharp increase in activity from 24 to 48 hours incubation.

4.1.2(ii) Comparison of temporal production of chitinase by T.harzianum genotypes

Chitinase activity was exhibited by all genotypes of *T. harzianum* (Fig 4.5). Genotype 3 differed to the other genotypes because initially activity was higher than with other genotypes and then it rapidly decreased with incubation time. The general trend of genotypes 1, 2 and 4 was an increase in chitinase activity. Genotypes 1 and 4 exhibited the highest activity; these are non-aggressive and aggressive compost colonisers, respectively. There was some similarity between the two non-aggressive genotypes (Th1 and 3) as both displayed a decrease in activity between 12 and 24 hours under inducing conditions.

4.1.2(iii) Comparison of temporal production of protease by T.harzianum genotypes

General protease activity was demonstrated by all four genotypes of *T. harzianum* (Fig 4.6). Although genotypes 2, 3 and 4 showed a general increase in protease activity, genotype 1 exhibited very different activity, which was initially high and then decreased. Azocasein, the protease substrate used for these assays, provided an indication of overall protease activity, however there are many classes of proteases and therefore two specific protease activities were investigated. Proteases Pr1 (chymoelastase) and Pr2 (trypsin-like protease) were followed as these enzymes have been characterised from *Metarhizium anisopliae* and have important role in entomopathology (St Leger *et al.* 1987a).

Clear differences were evident in the comparisons of Pr1-like and Pr2-like activities of *T. harzianum* genotypes (Fig 4.7 and 4.8). The aggressive genotypes (2 and 4) exhibited substantially higher Pr1-like and Pr2-like activities than the non-aggressive genotypes (1 and 3) after 12 hours under inducing conditions. By 24 hours of induction genotypes displayed similar levels of Pr1-like and Pr2-like activity (Fig 4.7b and 4.8b). Pr1-like activity was maintained, by all genotypes over 72 hours of incubation under inducing conditions, (Fig 4.7d). After reaching maximal activity after 24 hours, Pr2-like activity of genotypes 1, 2 and 4 gradually decreased with incubation time (Fig 4.8). In contrast genotype 3 continued to exhibit high Pr2-like activity over 72 hours incubation (Fig 4.8d).

In summary, aggressive genotypes revealed early and substantial chymoelastase and trypsin-like protease activity under conditions of induction. While non-aggressive genotypes demonstrated a delayed production of these enzymes and only reached activities equivalent to aggressive strains, 12 hours later. Pr2-like activity of the aggressive genotypes rapidly decreased after 24 hours under inductive incubation.

4.1.2(iv) Inhibition of protease activity of T.harzianum

Inhibition studies of Pr1-like and Pr2-like activity were performed by pre-incubation of samples with ethylenediaminetetraacetic acid (EDTA), iodoacetic acid (IAA), pepstatin, phenylmethylsulphonyl fluoride (PMSF) and leupeptin (Ac-Leu-Leu-Arg-hemisulfate) (all supplied by Sigma, UK). Controls were included of the carrier solvent for each inhibitor: water pH 8.5 (EDTA), water (IAA, leupeptin), dimethyl sulphoxide (DMSO) (pepstatin) and methanol (Beynon and Salvesen 1989).

Three strains from each *T. harzianum* genotype were tested for inhibition profile of Pr1-like activity. All indicated the same type of inhibition i.e. there was complete inhibition of Pr1-like activity by PMSF and slight inhibition (9.4%) by leupeptin (Fig 4.9 only data for strain T7 (Th2) shown). Due to limited supply of Pr2 substrate only one strain of each *T. harzianum* genotype was tested for inhibition of activity. Pr2-like activity of *T. harzianum* strain T7 was substantially inhibited by leupeptin. EDTA induced slight inhibition. Exposure to IAA and pepstatin had no effect on Pr1-like or Pr2-like activity.

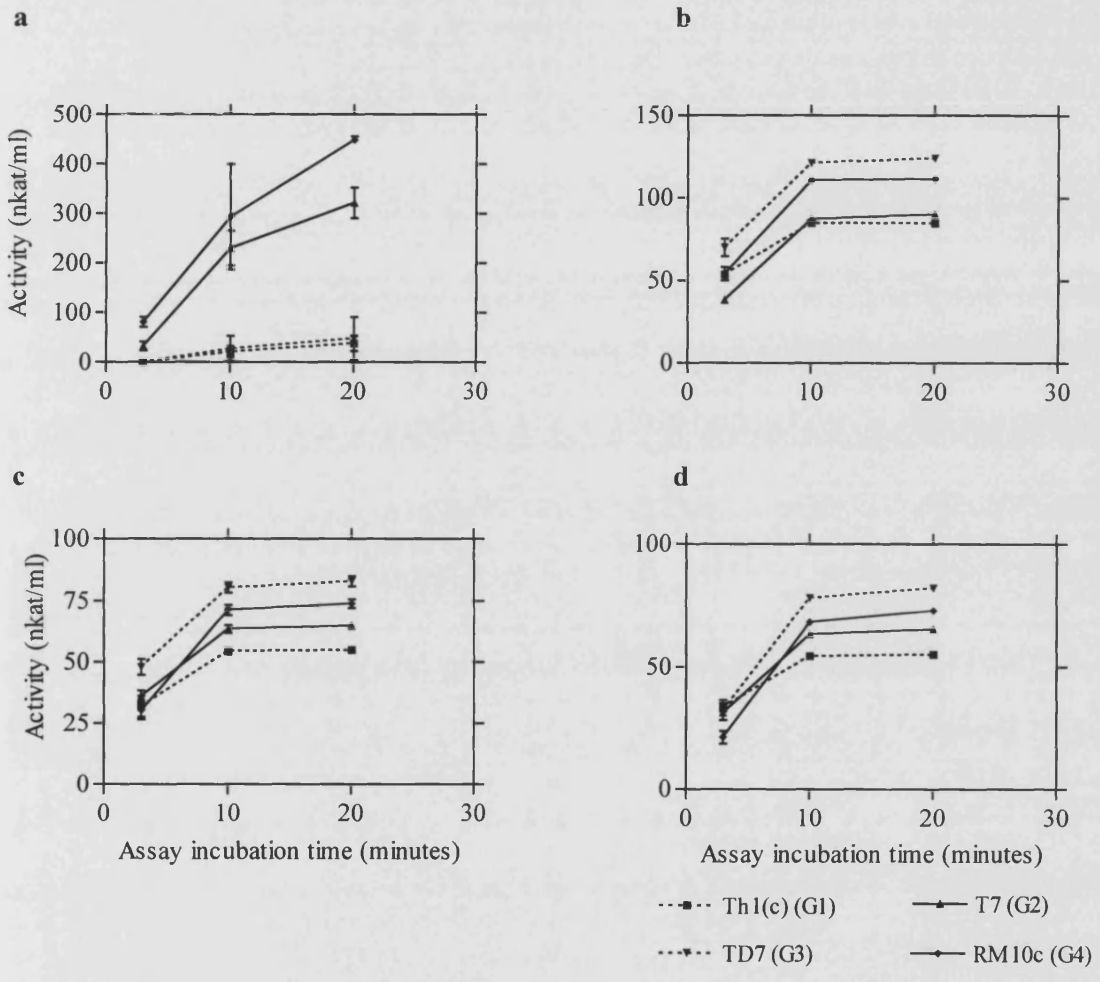


Figure 4.7: Pr1-like activity of *T. harzianum* genotypes in the presence of *Agaricus* cell walls: a) after 12 hours induction; b) 24 hours induction; c) 48 hours induction; d) 72 hours induction. Bars indicate SEM. Activity calculated as nkat/ml/μg protein.

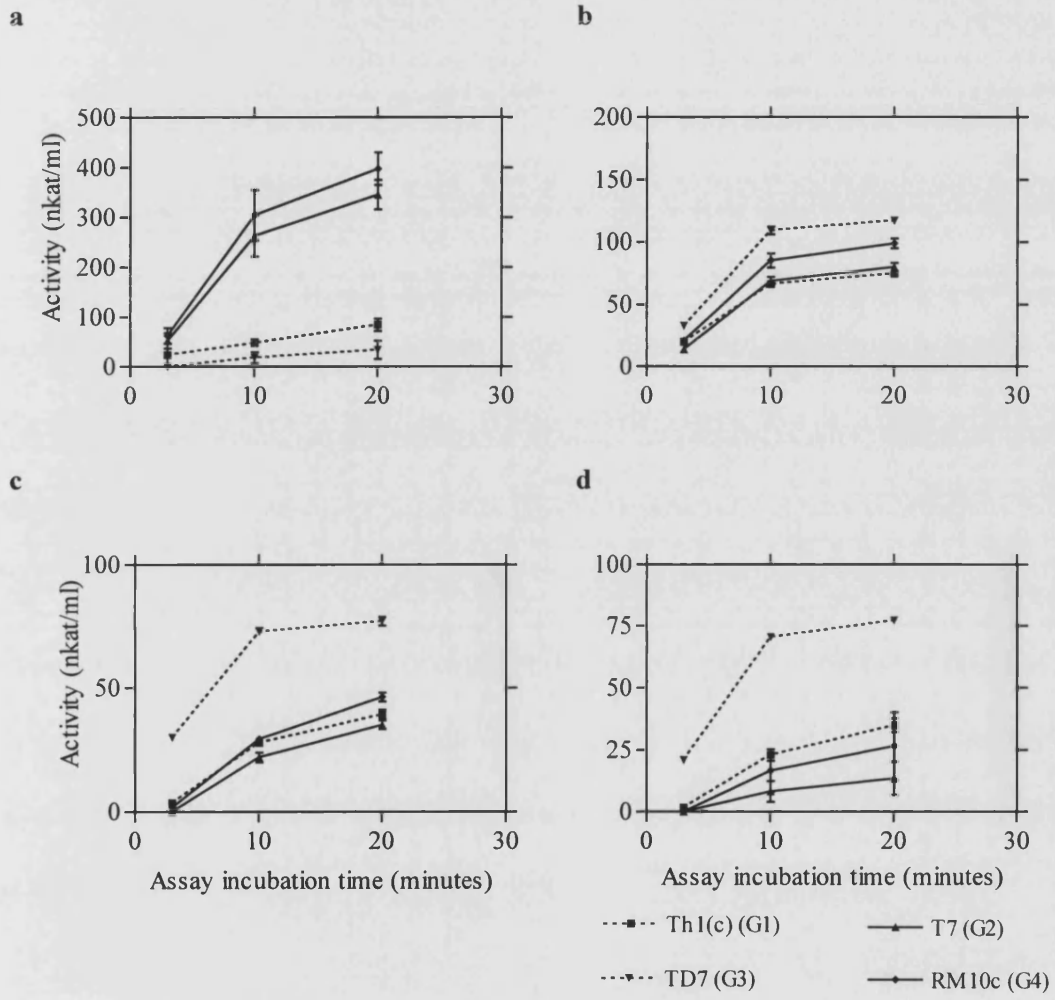
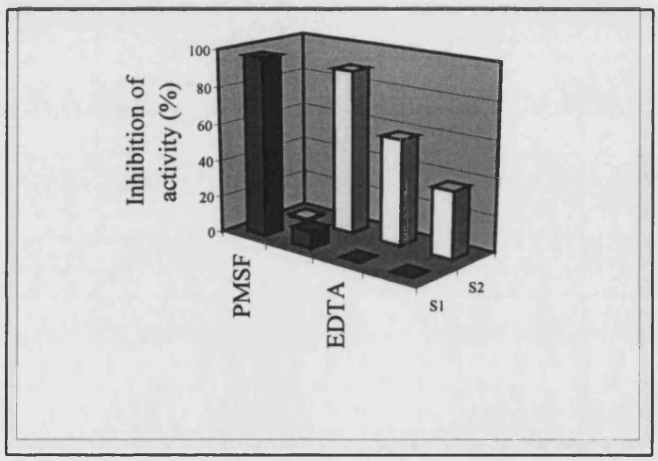


Figure 4.8: Pr2-like activity of *T. harzianum* genotypes in the presence of *Agaricus* cell walls: a) after 12 hours induction; b) 24 hours induction; c) 48 hours induction; d) 72 hours induction. Bars indicate SEM. Activity calculated as nkat/ml/μg protein.

Figure 4.9: Inhibitors of Pr1-like and Pr2-like activities of *T. harzianum* strain T7 (Th2).



4.1.2(v) Production of extracellular and cell wall-bound depolymerases by T. harzianum in the presence of two polymeric carbon sources

Trichoderma harzianum strain T7 (genotype 2) was cultured in the presence of 1% w/v *A. bisporus* cell wall material or 1% w/v crab shell chitin (Sigma). Crab shell chitin is an example of a possible cell wall-bound component in addition to its possible extracellular presence in the environment. Chitin, a known inducer of several mycoparasitic-related depolymerase including some chitinases and proteases of *T. harzianum* (Geremia *et al.* 1993), was used as an internal control. Cell wall extracts of *A. bisporus* provided a close approximation to the conditions exhibited *in situ*.

After an induction period of 24 hours, cell-free fluids and mycelial samples were collected. Mycelial samples (1g fresh weight quantities) were subjected to different conditions designed to dissociate enzymes attached cell walls. The mycelial samples were exposed to distilled water or phosphate buffer (50 mM, pH6.0) for the removal of depolymerases loosely attached to the cell wall. Exposure of mycelium to KCl (0.4M) or 1% w/v SDS enabled the dissociation of ionically or lipidic bound depolymerases, respectively. The mycelial samples and various washing solutions were shaken at 4°C for 2 hours and subsequently, samples of supernatant were removed. Supernatants were dialysed against 25 mM citrate buffer (pH 6.0) at 4°C for 12 hours before enzyme assays.

Extracellular laminarinase activity was higher (*ca.* 1.65-fold) in the presence of *A. bisporus* cell walls than crab shell chitin (Fig 4.10). Extracellular activity represented the highest activity on both carbon sources. However, in the presence of *A. bisporus* cell walls, abundant cell wall-bound enzyme activity was detected (when mycelium was washed in KCl, SDS or phosphate buffer.

Extracellular chitinase activity was substantially higher (*ca.* 9-fold) in the presence of *A. bisporus* cell walls (Fig 4.11). The majority of chitinase activity was extracellular and comparatively little chitinase activity appeared to be bound to the cell wall.

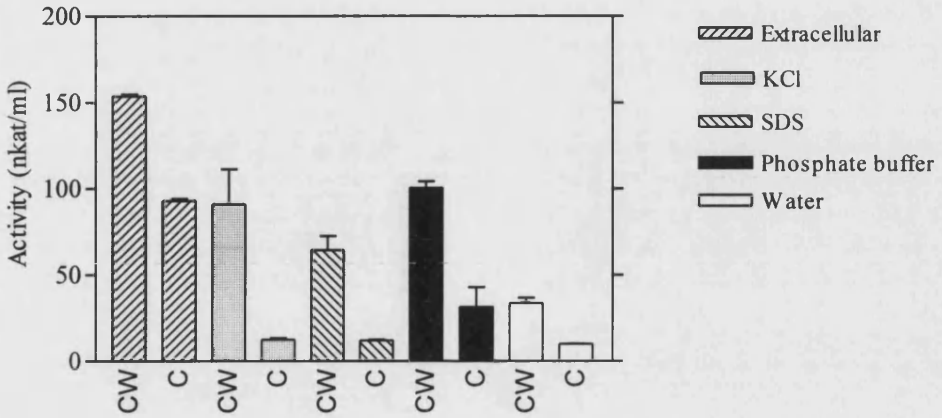


Figure 4.10: Production of extracellular and bound laminarinase by *T. harzianum* strain T7 (Th2) on two polymeric carbon sources. Bars indicate SEM. CW, indicates cultures supplemented with *A. bisporus* cell wall; C, crab shell chitin supplement. Activity calculated as nkat/ml/g dry weight mycelium.

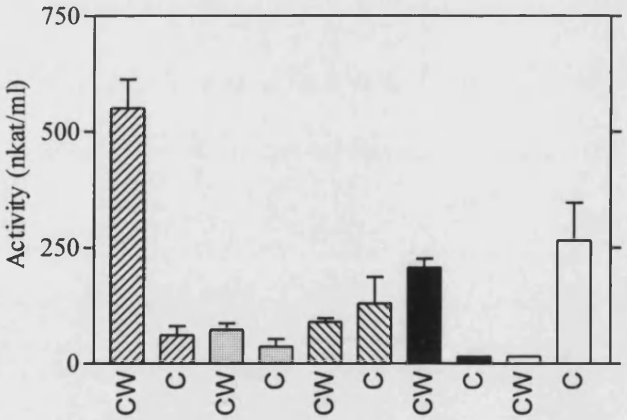


Figure 4.11: Production of extracellular and bound chitinase by *T. harzianum* strain T7 (Th2) on two polymeric carbon sources. Bars indicate SEM. Activity calculated as nkat/ml/g dry weight.

4.1.3 Comparison of extracellular enzyme profiles for T. harzianum genotypes

API-ZYM™ strips (api, Bio Merieux, France) which contain substrates for some 19 enzymes, provided a rapid method for the comparison of enzyme profiles of several strains of *T. harzianum*. The results determined were semi-quantitative as colour change was compared to a reading scale provided. Culture filtrate samples from *T. harzianum* strains grown in the presence of *A. bisporus* cell walls were removed after 24 hours incubation. Samples (25 µl per well) were applied to semi-quantitative API-ZYM™ strips, which were incubated at 37°C for 1 hour. Hydrolysis products were detected by the addition of 50µl of reagent ZYM-A and ZYM-B to each well. Activity was determined by the colour intensity and measured against a reading scale supplied by the manufacturers. The enzyme activities investigated included: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystein arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

In general the enzyme profiles were similar for all *T. harzianum* genotypes (Fig 4.12). However, subtle differences were evident. The aggressive genotypes (2 and 4) revealed higher activities of alkaline and acid phosphatase, esterase lipase and naphthol. All genotypes except genotype 2, produced leucine arylamidase activity. Non-aggressive genotypes (1 and 3) were unique in the production of α-glucosidase activity. Genotype 4 (aggressive) was the only genotype to exhibit esterase, α-galactosidase and trypsin acitivity. All genotypes displayed β-glucosidase and *N*-acetyl-β-glucosaminidase activity, however genotype 2 exhibited substantially higher activities for both enzymes. Several enzyme activities were not detected in any *T. harzianum* filtrates and these included: valine arylamidase, cystein arylamidase, chymotrypsin, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase.

The non-aggressive genotypes (1 and 3) exhibited almost identical extracellular profiles with identical enzyme types and very similar level of activity for each enzyme, although genotype 3 tended to display higher activities (Fig 4.12a,c). Enzyme profile for genotype 2 (aggressive) was less complex than other genotypes, but all activities were high (Fig 4.12b). Genotype 4 (aggressive) demonstrated a more complex enzyme

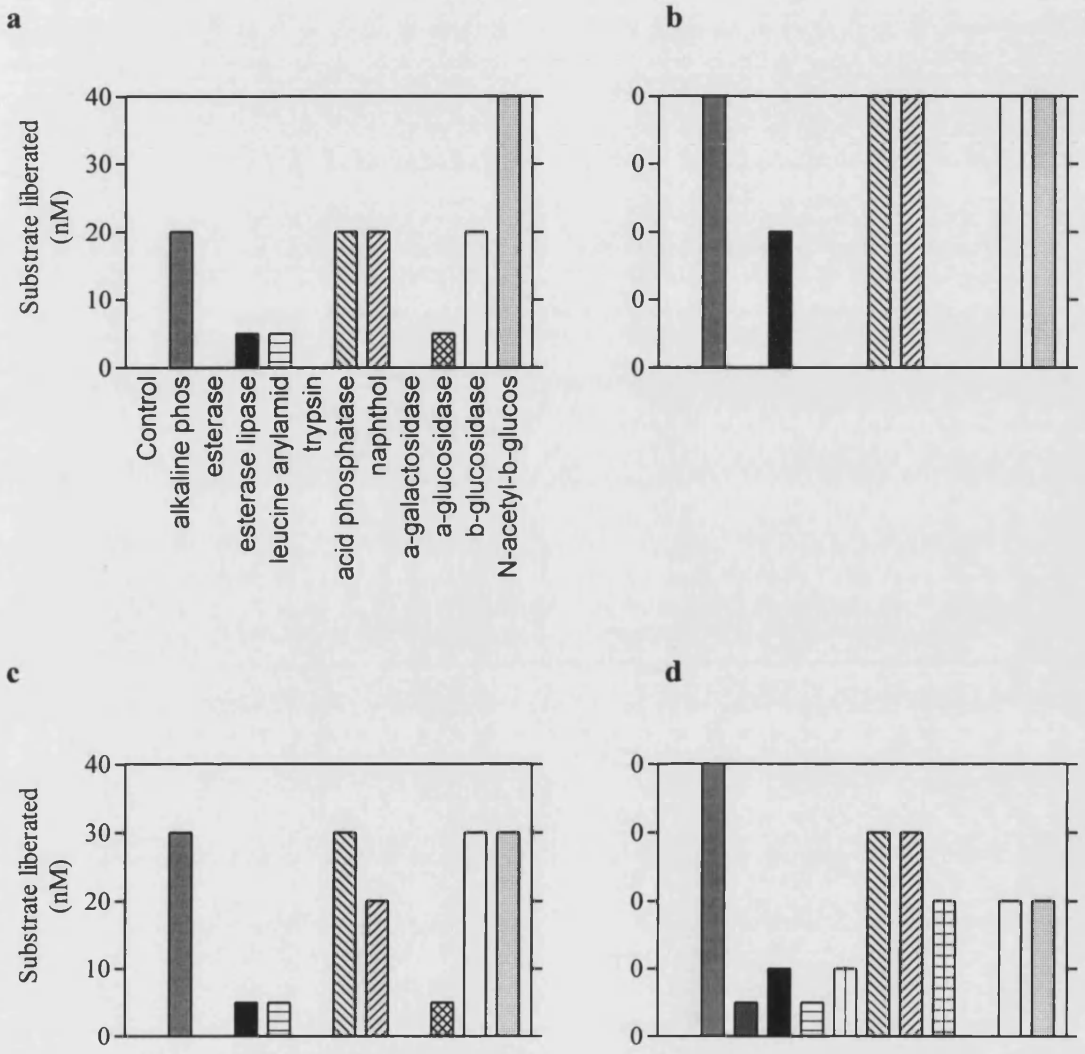


Figure 4.12: Extracellular enzyme profiles for *T. harzianum* genotypes cultured in the presence of *Agaricus* cell walls: a) Th1(c) (Th1); b) T7 (Th2); c) TD7 (Th3); d) RM10casing (Th4).

profile than all other genotypes (Fig 4.12d).

4.1.4 Isoforms produced by T.harzianum genotypes in vitro in the presence of A.bisporus cell walls

Molecular techniques have enabled the classification of four genotypes of *T. harzianum* (Muthumeenakshi *et al.* 1994, 1998; Ospina-Giraldo *et al.* 1997a, 1997b; Chen *et al.* 1997; Castle *et al.* 1997). To be able to distinguish genotypes by mycoparasitism related components could be extremely important in the diagnosis of strains of *T. harzianum* aggressive towards *A. bisporus* and particularly useful in understanding possible antagonistic mechanisms. Therefore a comprehensive study of depolymerase isoforms triggered under mycoparasitism-like conditions was conducted. The study required cell-free samples of culture fluids from *T. harzianum* grown in the presence of *A. bisporus* cell walls. Total protein samples from culture fluids were separated according to isoelectric points and specific activities determined. The technique was capable of processing many samples and therefore three strains from each genotype could be analysed.

Samples of culture filtrates were collected after 12 and 24 hours under inducing conditions. Total protein content (Materials and Methods 2.3.7) was determined and volumes containing 100 μg protein were flash frozen and lyophilised. Samples were applied to 5% Ampholine PAG plate gels (Pharmacia Biotech) in 10 μl sterile distilled water according to manufacturer's instructions (Materials and Methods 2.3.3). Proteins were separated according to iso-electric points.

4.1.4(i) Isoforms of laminarinase

Laminarinase activity was detected by incorporation of Remazol Brilliant Blue (RBB) - curdlan (Loewe Biochimica GmbH) to the activity gel. The dye-linked substrate was supplied at 4mg/ml. The substrate (4ml) was incubated at 75°C and was mixed with 1ml of hot agarose solution (5% agarose in 50 mM MES, pH 5.5). Activity gels were cast using polyacrylamide gel plates with 1 mm spacers. Gels were cut to size and placed directly on the IEF gel; contact was ensured by removal of all air bubbles. The gel was then incubated at 37°C for 30 minutes. Agarose overlays were removed and destained in 50 mM acetate buffer (pH 5.4) – ethanol (96%), 1:2 (v/v). Activity gels were preserved by the addition of 5% trichloroacetic acid to the destain (Biely *et al.* 1985a; b). Gels were incubated in

preservation solution for several hours at room temperature to denature enzymes. Finally, gels were blotted dry and sealed in plastic with glycerol and stored at 4°C.

All genotypes produced more than one isoform with laminarinase activity. *T. harzianum* strains within genotypes displayed an identical isoform pattern. Genotypes 1, 2 and 3 exhibited laminarinase isoforms with isoelectric points (pI) of 4.41, 4.6 and 4.83 (Fig 4.13). The isoform with pI 4.83 appeared to be the major isoform (based on prominence of the clearing zone). Genotype 4 produced three laminarinase isoforms with similar pI values to the other genotypes; pI 4.72, 4.85 and 5.04 were all detected from genotype 4 filtrates. The main isoform secreted by genotype 4 in the presence of *A. bisporus* cell walls had a pI of 6.25.

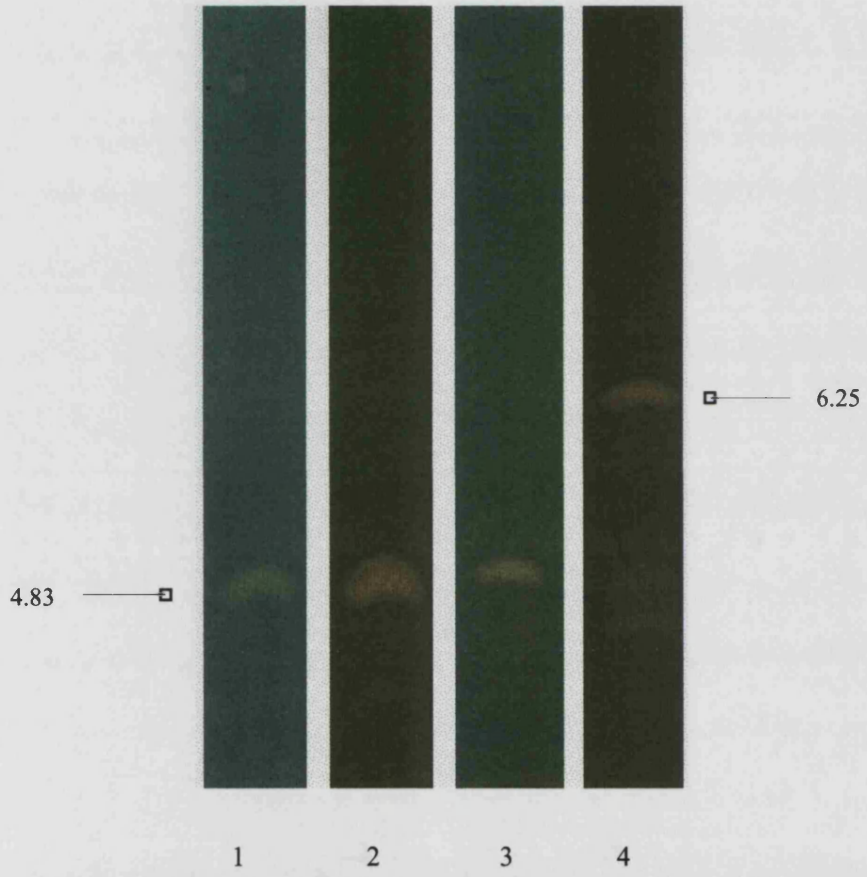
After 12 hours culture all the above mentioned isoforms were produced, but after 24 hours under inducing conditions only the major isoforms (pI 4.83 and pI 6.25) were visible with clarity (Fig 4.13) and the activities of other isoforms had reduced.

4.1.4(ii) Isoforms of chitinase

Detection of chitinase activity was accomplished using carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV) (Loewe Biochimica GmbH). The substrate (4mg/ml) was added to hot agarose solution (as described 4.14(i)). Gels were cast in polyacrylamide gel plates with 1 mm spacers, cut to size and applied to IEF gel. The gels were incubated at 37°C for 30 minutes. Subsequently the overlays were removed, destained and preserved using the solutions and methods described above (section 4.1.4(i)).

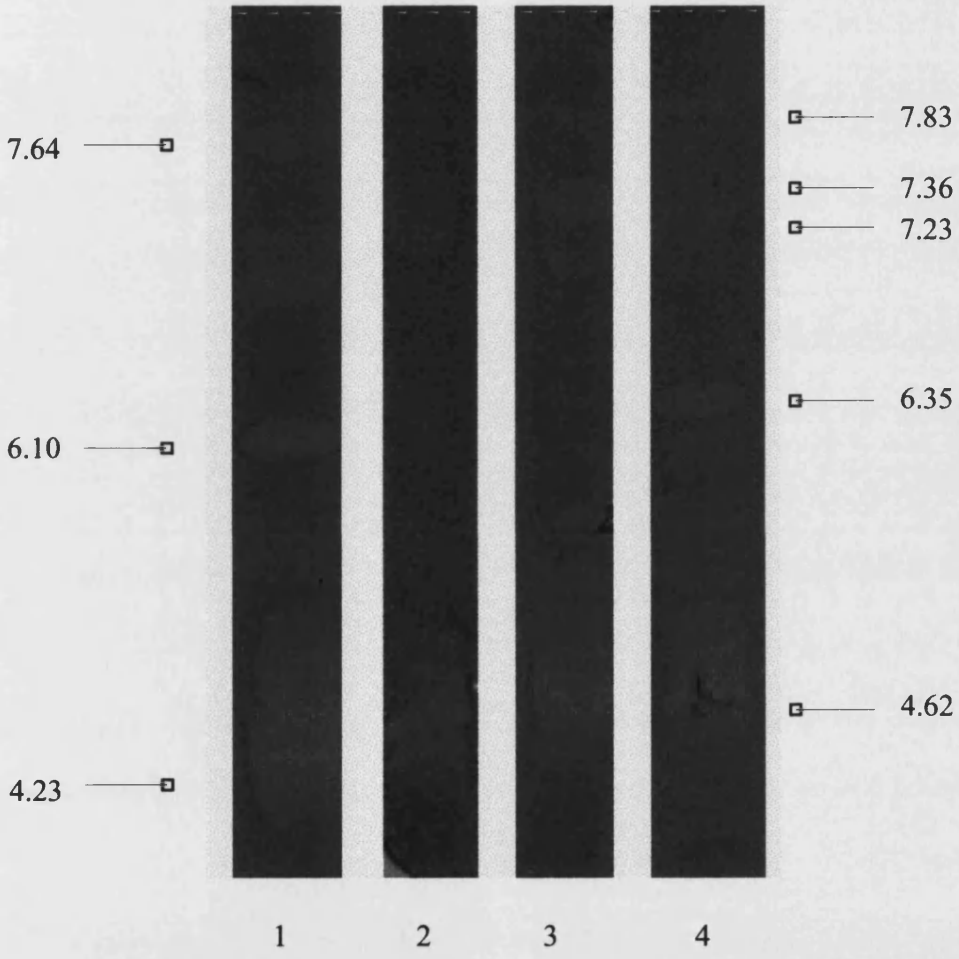
The number of chitinase apparent isoforms increased with induction time. After 12 hours, genotype 1 (non-aggressive) produced isozymes with pI 4.46 to 4.84, 6.22 and 6.92. Genotypes 2 and 3 produced a range of isoforms with pI's 4.6 to 5.66. Genotypes 2 and 4 (aggressive) were unique in the secretion of a doublet of isoforms with pI 7.23 and 7.36. Genotype 4 also produced chitinase isozymes with pI of 4.65, 5.03 and 5.22. Thus there was significant overlap of isoforms with acidic iso-electric points.

Figure 4.13: Isoforms of laminarinase produced by *T. harzianum* genotypes in the presence of *A. bisporus* cell walls.



Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); RM10c (Th4).
Activity after 24 hours induction. Values denote pI as determined from a calibration curve.

Figure 4.14: Isoforms of chitinase produced by *T. harzianum* genotypes in the presence of *A. bisporus* cell walls.



Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4). Activity after 24 hours induction. Values denote pI as determined from a calibration curve.

Induction for 24 hours resulted in the appearance of many additional isoforms (Fig 4.14). The aggressive genotypes (2 and 4) secreted many isoforms with pI values in the range 4.62 to 6.35 and also an isoform of pI 7.83 was detected. An isoform with pI 6.35 was prominent for genotype 4 strains. Genotype 2 continued to secrete isoforms with pI 7.23 and 7.36, while genotype 4 only exhibited activity with pI of 7.23.

Non-aggressive genotypes (1 and 3) induced for 24 hours had similar chitinase isoform profiles (Fig 4.14). Both genotypes produced chitinase isozymes with acidic pI's in the range 4.23 to 5.20 and alkaline pI isoforms within the range 7.00 to 7.45. In addition genotype 1 secreted isozymes with iso-electric points of 6.1, 6.8 and 7.64, all of which appeared to be prominent isoforms.

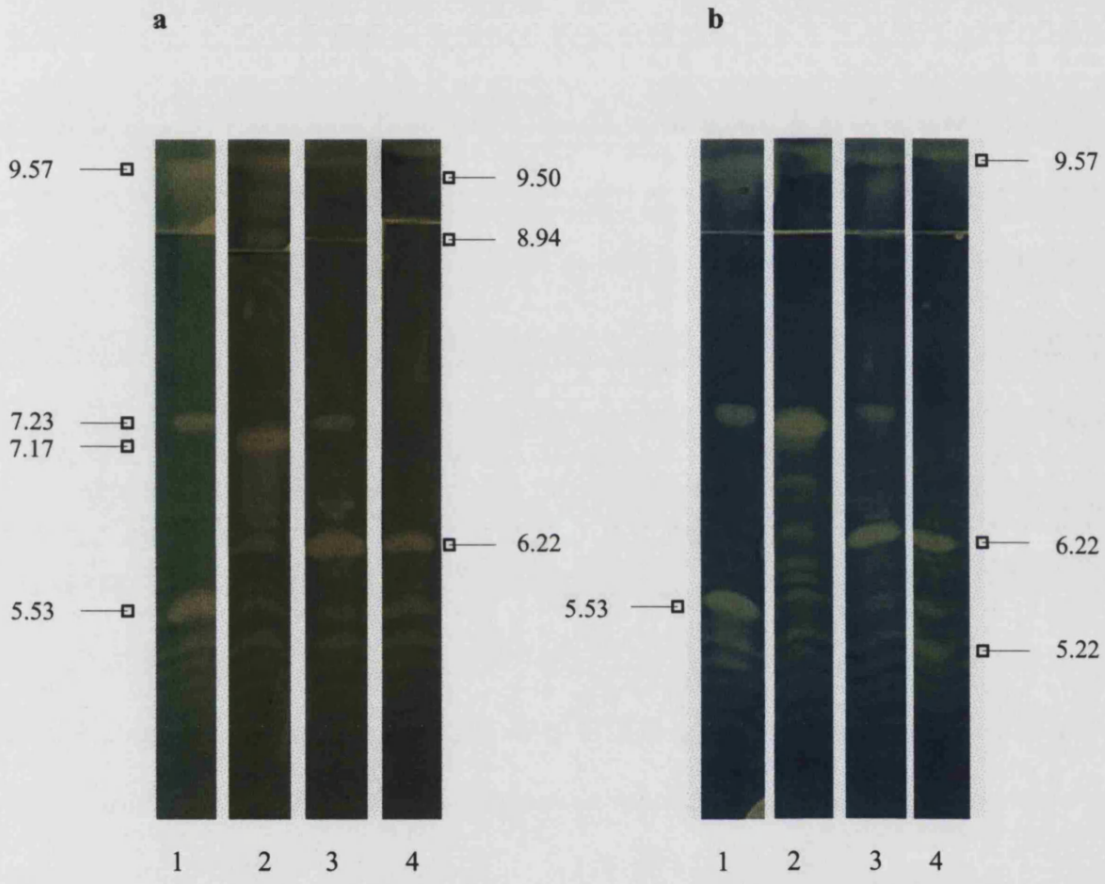
In summary, the aggressive genotypes (2 and 4) produced chitinase isoforms with a broad range of iso-electric points. In contrast, the non-aggressive genotypes (1 and 3) secreted isoform clusters with acidic or alkaline pI's. However, genotype 1 produced one isoform which had a pI value between those of the acidic and alkaline clusters, an isoform with pI 6.1.

4.1.4(iii) Isoforms of protease

Remazol Brilliant Blue (RBB)-gelatin (Loewe Biochimica GmbH) was used to detect protease activity. The substrate was supplied in solution (4mg/ml) and 4 ml was added to hot agarose solution (as described 4.1.4(i)). Gels were cast in polyacrylamide gel plates with 1 mm spacers, cut and placed directly on IEF gel surface. After incubation at 37°C for 30 minutes, the activity gels were destained and preserved as described above (section 4.1.4(i)). The destain step was monitored closely as the solution had adverse effects on non-hydrolysed substrate if exposed to destain for extended periods of time.

Genotypes 2 and 3 appeared to produce a higher number of isoforms after 12 hours induction than after 24 hours (Fig 4.15). The longer culture period resulted in reduced activity of some isoforms, while other isoform activities disappeared. While many minor isoforms were produced by all genotypes including isoform pI 5.53, some of the major isozymes displayed overlap in their pI values. After 12 hours induction genotypes 2,3 and 4 all secreted an isoform with pI 6.22 (Fig 4.15a). Genotypes 3 and 4 produced a highly

Figure 4.15: Isoforms of protease produced by *T. harzianum* genotypes in the presence of *A. bisporus* cell walls.



Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4).
(a) 12 hours induction. (b) 24 hours induction. Values denote pI as determined from a calibration curve.

alkaline isoform with pI 9.57. Genotypes 1 and 2 produced an isoform with a pI of 7.23. Genotype 3 secreted many additional protease isoforms including a group with highly alkaline pI's in the range 8.94 to 9.50 and a dominant isoform with pI 7.17.

Exposure to inducing conditions for 24 hours resulted in the loss of some minor isoforms, in particular those of genotypes 2 and 3 (Fig 4.15b). After 24 hours all genotypes secreted an alkaline isozyme of pI 9.57. Genotype 1 continued to secrete the major isoforms pI 5.53 and 7.23, while genotype 3 was reduced to one prominent isoform of pI 7.17. Genotypes 2 and 4 (aggressive) persisted in the secretion of a principal isoform with pI 6.22. In contrast, genotype 3 had greatly reduced activity at pI 6.22. Th4 was unique in secreting isoforms with iso-electric points of 5.22 and 5.53.

In summary, the aggressive genotypes (2 and 4) were unique in the secretion of an isoform of pI 6.22 after 24 hours of culture. Genotypes 1, 2 and 3 exhibited similar isoforms in the pI range 7.17 to 7.36. Protease activities of minor isoforms decreased with incubation time, while most dominant isoforms were maintained.

4.2 Discussion

While exhibiting characteristics of ruderal fungi, such as high growth rate and production of vast numbers of reproductive propagules on simple carbon sources (Pianka 1970), *T. harzianum* also displayed attributes commonly associated with more combative fungi. The production of an extensive range of depolymerases is one example (Widden and Scattolin 1988). In this research the introduction of *A. bisporus* cell walls as a sole carbon source after a period of starvation demonstrated the ability of *T. harzianum* to secrete high levels of a wide range of depolymerases necessary for *A. bisporus* cell wall degradation. Chitinases, laminarinases and proteases were secreted by all genotypes regardless of their ability to reduce mushroom yields.

4.2.1 Induction of T. harzianum cell wall depolymerases on exposure to A. bisporus cell walls

All *T. harzianum* genotypes demonstrated rapid production of depolymerases of potential relevance to the degradation of *A. bisporus* cell walls. High activities of chitinase and laminarinase were detected for all genotypes. However Th2 and Th4 displayed the most rapid and high production of protease activities determined as chymoelastase and trypsin-like by substrate specificities. Sequential secretion of depolymerases reflected the possible architecture of the *A. bisporus* cell wall extract.

The introduction of *A. bisporus* cell walls was designed to simulate conditions of mycoparasitism. Attention should be drawn to the finely divided state of the lyophilised cell wall preparation which can only give an approximation of the native polymers present, since the mechanical techniques used in the preparation may alter the structure of the cell wall. Thus chitin fibrils, usually embedded in protein beneath a β-glucan layer (Michalenko *et al.* 1976), may be more accessible in cell wall extracts than during a mycoparasitic interaction *in situ*. Sequential secretion as determined *in vitro* may not accurately represent the order of enzyme secretion during interactions between two viable fungi in mushroom compost.

Measurements of total activity concealed some important differences at the level of isozymes; however the determination of total activity was a relatively rapid and inexpensive method to compare the depolymerase activities of several strains.

Optimisation of the assays for total activity for individual depolymerases was achieved by partial characterisation of pH, temperature and assay incubation time optima.

All genotypes, with the exception of Th3, exhibited an increase in extracellular chitinase activity with time. Therefore exposure to *A. bisporus* cell walls induced the secretion of chitinase from both aggressive and non-aggressive strains of *T. harzianum*. Th3 appeared to be triggered very rapidly as chitinase activity was apparently maximal before the first sample collection time after 12 hours induction.

Extracellular laminarinases were produced by all genotypes, which was not unexpected as many fungi produce basal levels of certain laminarinases constitutively (de la Cruz *et al.* 1993). The apparent up-regulation of laminarinase activity with time was presumably a response to β-1,3-glucans in *A. bisporus* cell walls.

Substantial differences were detected between aggressive and non-aggressive genotypes of *T. harzianum* in protease activities similar to Pr1 (chymoelastase) and Pr2 (trypsin-like) of *Metarhizium anisopliae* (St. Leger *et al.* 1987a). Aggressive genotypes (Th2 and Th4) exhibited rapid secretion of Pr1-like and Pr2-like activity at substantially higher levels than non-aggressive *T. harzianum*, typically 12.5-fold and 7.3-fold for Pr1-like and Pr2-like activity respectively. This may suggest a role for protease(s) linked to aggressiveness. Protease activity is often triggered early after recognition events (Flores *et al.* 1997) and some proteases can be stimulated by chitin or chitin-containing substrates (Geremia *et al.* 1993). *T. harzianum* Pr1-like and Pr2-like activities in the presence of *A. bisporus* cell walls indicated similarities with the entomopathogenic fungus *M. anisopliae* which secretes these proteases in the presence of host insect exoskeleton (Paterson *et al.* 1994). This activity is also very similar to that of Prb1 (a chymoelastase), which was secreted by *T. harzianum* in the presence of *R. solani* cell walls (Geremia *et al.* 1993). Prb1 and Pr1 are completely inhibited by phenylmethylsulphonyl fluoride (PMSF) which is indicative of serine proteases (St Leger *et al.* 1987; Geremia *et al.* 1993). Almost complete inhibition (*ca.* 97%) of the Pr1-like activity of *T. harzianum* by PMSF in this work was consistent with that of Prb1 and Pr1.

Proteases have been reported for their importance in the degradation of fungal host cell walls by mycoparasites (Elad *et al.* 1982; Ridout *et al.* 1988; Geremia *et al.* 1993). The up-regulation of Prb1 and the introduction of more gene copies increased the biocontrol effect by *T. harzianum* over *R. solani* (Flores *et al.* 1997). Proteins and β-glucans often comprise the matrix of the cell wall and adhere to the fibrillar component, thus conferring structure and strength to the cell wall (Michalenko *et al.* 1967). Early induction of proteases (*in vitro* sequential secretion, this work) may allow them to permeate the cell wall matrix and thus expose chitin fibrils; subsequent or simultaneous induction of chitinases could then result in more rapid and efficient degradation of host cell walls. The difference between genotypes was exhibited only at the earliest sample (12 hours induction) suggesting the importance of rapid production of proteases and a link with possible mycoparasitism-like behaviour of aggressive strains of *T. harzianum*. Genotype-specific increase in Pr1-like and Pr2-like activity could perhaps be regulated by a specific recognition event resulting in their induction. An example of a recognition trigger may involve a lectin-carbohydrate complex (Elad *et al.* 1983b and Appendix). Interestingly, *A. bisporus* secreted a serine protease during mycelial growth in mushroom compost (Burton *et al.* 1997). *Verticillium fungicola* var. *fungicola*, the causal agent of dry bubble disease of the commercial mushroom, also has demonstrated protease activity (possibly serine) in the presence of *A. bisporus* cell walls (Kalberer 1984). This pathogen is invasive and its destructive internal growth has led to suggestions of the involvement of serine protease in the degradation of host organelles (Dragt *et al.* 1996).

Pr2-like activity of Th2 (T7) differed slightly from Pr2 of *M. anisopliae* in sensitivity to inhibitors. Substantial inhibition was caused by leupeptin, suggestive of a trypsin-like protease and this was also reported for Pr2 of *M. anisopliae* (St Leger *et al.* 1987a). However, *T. harzianum* Pr2-like activity was also inhibited by the chelator EDTA (indicative of metallo-protease) and pepstatin (inhibitor of aspartic proteases) and not by PMSF like Pr2 of *M.anisopliae* (St. Leger *et al.* 1987). This suggests the Pr2-like activity of *T. harzianum*, while similar to Pr2 of *M. anisopliae* in substrate specificity (Bz-Phe-Val-Arg-NA), is not produced by the same type of protease but by several different types, resembling combined metallo-proteases, aspartic and trypsin proteases. In contrast, Pr2 activity of *M. anisopliae* is clearly the result of a classic trypsin protease (St. Leger *et al.* 1987). Nevertheless, *T. harzianum* exhibited Pr2-like activity and since Pr2 of *M.*

anisopliae has a role in parasitism it follows that the *T. harzianum* Pr2-like activity may be important for mycoparasitism of *A. bisporus*.

The sequential secretion of depolymerases (Fig 4.3) also supported a possible role for proteases during an antagonistic interaction between *T. harzianum* and *A. bisporus*. A rapid and simultaneous increase in activity of protease and chitinase from an aggressive strain of *T. harzianum* was detected within the first 12 hours of induction. Geremia *et al.* (1993) reported that the protease Prb1 was induced by the presence of chitin or chitin-containing complexes and likewise De la Cruz *et al.* (1992) reported that chitinases were induced by the same substrates. This suggests the need for simultaneous degradation of these closely associated substrates, which would require coordinate induction of depolymerases. Possible partial detachment of the outer β-1,3-glucan layer of *A. bisporus* cell wall extract may explain the lag in laminarinase activity. Thus when antagonising intact *A. bisporus*, the laminarinase activity of *T. harzianum* may be produced initially in order to degrade the β-glucan layer that offers some protection to the chitin fibrils. However with a cell wall preparation the accessibility of chitin and protein are likely to be increased and result in the secretion by *T. harzianum in vitro* in the order of protease and chitinase, and then laminarinase. Regardless of the sequential appearance, all the necessary extracellular depolymerases were induced, rapidly after exposure to *A. bisporus* cell walls. In other systems sequential secretion of cell wall depolymerases is evident, for example *Rhizoctonia cerealis* exhibited enzyme activity in the order of arabanase, followed by xylanase and then laminarinase in the presence of cell walls of wheat seedlings or wheat straw (Cooper *et al.* 1988). The structure of the host cell wall was of direct relevance to the enzyme activities produced since Cooper *et al.* (1988) reported differing levels of depolymerase activities of *R. cerealis* on exposure to dicotyledonous cell walls, in particular low xylanase activity and high β-D-galactosidase activity.

Extracellular and cell wall-bound laminarinase activity produced by *T. harzianum* in the presence of crab shell chitin may represent constitutive or coordinate production. Elad *et al.* (1982) reported that laminarinases were often produced constitutively in the presence of several carbon sources including chitin. Here laminarinase activity was induced dramatically on exposure to *A. bisporus* cell walls that contained the substrate β-1,3-glucan. This increased laminarinase activity (typically 1.65-fold for extracellular, 7.14-fold for ionically and lipidically and 3.33-fold for loosely cell wall-bound activities) on

exposure to *A. bisporus* cell walls suggests the exposure of potential substrates by the synergistic action of combined depolymerase activities leading to enhanced cell wall degradation and higher individual depolymerase activities (*cf.* crab shell chitin). Lipidically and ionically cell wall-bound activities may allow localised host cell wall degradation when *T. harzianum* is in contact with the host.

The majority of chitinase activity was detected as extracellular and exposure to *A. bisporus* cell walls induced a substantial increase (*ca.* 9-fold) in extracellular activity compared to exposure to chitin. This could be suggestive of a recognition event (Inbar and Chet 1995) since in this work *T. harzianum* secreted lectins in liquid cultures (see Appendix B.1) or due to varying levels of derepression dependent on the intractability of the chitin found in *A. bisporus* cell walls and crab shell chitin. The production of extracellular chitinase suggested that host cell wall degradation could occur at distance. Since cell wall-bound activities were relatively low (*cf.* extracellular) perhaps these activities were less important during the initial steps of mycoparasitism, particularly if the host was recognised at distance. Loosely bound chitinase released by phosphate buffer was the highest cell wall-bound activity and could possibly represent protein recently secreted *via* the secretory pathway (Alberts *et al.* 1989). Here chitinase activity bound loosely and lipidically to the cell wall was higher when exposed to crab shell chitin than to *A. bisporus* cell walls and may have been indicative of more than one regulatory system. De la Cruz *et al.* (1992) found that only one of three endochitinases secreted by *T. harzianum* in the presence of crab shell chitin, could be detected when exposed to *B. cinerea* cell walls as a sole source of carbon.

Cell wall degradation may occur in a rapid uncontrolled manner such as during necrotrophic mycoparasitism or by the proposed tightly regulated degradation exerted by a biotroph, for example *Colletotrichum lindemuthianum* during the initial biotrophic phase, exhibited localised host cell wall degradation restricted to sites of penetration (O'Connell *et al.* 1985). Depolymerases may be controlled by protein-DNA complexes, for example a carbon catabolic repressor protein (Cre1) binds to the promoter sequence of a 42 kDa endochitinase (Ech42) gene of *T. harzianum* during non-mycoparasitic conditions but is outcompeted under conditions of mycoparasitism (Lorito *et al.* 1996b).

4.2.2 Extracellular enzyme profiles of T. harzianum genotypes exposed to A. bisporus cell walls

Extracellular enzyme profiles of *T. harzianum* genotypes using API-ZYM™ strips revealed genotypic and geographical differences. The basic enzyme profile resulting from the exposure to *A. bisporus* cell walls was demonstrated by Th2 (T7) which exhibited a simpler profile than the other genotypes. Aggressive genotypes of *T. harzianum* exhibited higher activities of acid and alkaline phosphatase than did non-aggressive genotypes. Phosphatase activity of *T. harzianum* origin may afford a competitive role since efficient phosphatase activity may allow *T. harzianum* to utilise available phosphates in compost and deprive *A. bisporus* of this vital element. *A. bisporus* displayed phosphatase activity which was not associated with fruiting, suggesting an involvement in obtaining phosphates for metabolism (Wood and Goodenough 1977). The higher esterase lipase activities by aggressive than by non-aggressive genotypes in the presence of *A. bisporus* cell wall extract may be relevant to mycoparasitism in view of the importance of some fatty acids in the reproduction of *A. bisporus* (Cruz *et al.* 1997). All genotypes exhibited β-glucosidase and *N*-acetyl-*β*-glucosaminidase activities, suggesting efficient degradation of disaccharides before rapid uptake in a permeable form.

The non-aggressive genotypes were almost identical in extracellular enzyme profile but differed in the amount of activities exhibited. Th3 typically displayed higher activities than did Th1, which may be linked to the greater saprophytic growth ability of Th3 compared to that of Th1 (see Chapter 5). Non-aggressive genotypes also exhibited α-glucosidase activity that was not produced by the aggressive genotypes, indicating that this activity was not required for aggressive behaviour towards *A. bisporus*.

Th4 displayed a more complex extracellular enzyme profile than the other genotypes with the additional presence of esterase, trypsin and α-galactosidase activities. Geographical origin may explain these differences; Th4 originated from North America, while the other genotypes are indigenous to Europe (Seaby 1996a). The classification of the four genotypes has been achieved with molecular techniques (Muthumeenakshi *et al.* 1994, 1998; Romaine *et al.* 1997; Ospina-Giraldo *et al.* 1997; Chen *et al.* 1997; Castle *et al.* 1998) and differences detected in the enzyme profiles of the genotypes supported the broad conclusions of the molecular techniques. Several classes of protease were not detected in the culture fluids but this could have reflected the single time of sampling (24 hours). Th4

was unique in production of trypsin activity, which may have an involvement in mycoparasitism and this would support the role of *T. harzianum* proteases in the mycoparasitism of *R. solani* claimed by Geremia *et al.* (1993) and Flores *et al.* (1997). Perhaps Th4 exhibited trypsin activity (Pr2-like activity) for a continued period of time being particularly aggressive towards *A. bisporus*.

4.2.3 Depolymerase isoforms of T. harzianum induced in the presence of A. bisporus cell walls

Isozyme profiles of the individual classes of depolymerases enabled a more in depth investigation of the complex nature of such enzymes. Although measuring the total activity of *A. bisporus* cell wall-degrading depolymerases revealed few significant differences, when the activity of the isoforms was targeted some quite striking genotype and geographical differences were apparent.

Laminarinase isoform profiles provided a characteristic with which genotypes of *T. harzianum* could be geographically classified. European genotypes (1, 2 and 3) produced identical acidic isoforms of laminarinase, while the North American genotype secreted a dominant more alkaline isoform (*cf.* other genotypes). Vazquez-Garciduenas *et al.* (1998) demonstrated that *T. harzianum* secreted two laminarinase isoforms (pI 6.6 and 6.8) that were consistently induced by cell walls of *M. rouxii*, *N. crassa*, *R. solani* and *S. cerevisiae*. In this work the laminarinase isoforms induced by *A. bisporus* cell walls were very different to those reported by Vazquez-Garciduenas *et al.* (1998). De la Cruz *et al.* (1995a) described the secretion of several laminarinase isoforms in the presence of chitin (pI 5.3, 6.2 and >7.4). In this study, isoforms secreted by Th4 are the most similar to those previously reported by De la Cruz *et al.* (1995a).

This apparent geographical difference was distinctive but would not allow classification of aggressive and non-aggressive genotypes. Nevertheless, the technique could be used to monitor (superficially) the dissemination of such genotypes. As research continues to contribute to the understanding of the green mould problem it may be important to have a method of distinguishing between genotypes of different continents.

Chitinase isoforms were produced that also distinguished *T. harzianum* genotypes (see Table 4.1). The aggressive genotypes (2 and 4) exhibited unique isoforms and these were detected after 12 and 24 hours induction. De la Cruz *et al.* (1992) reported *T. harzianum*

Table 4.1 Summary of genotype-distinguishing depolymerase isoforms.

Depolymerase	*T. harzianum* Genotype	Isoelectric point	Previously reported
Laminarinase	Th1	4.83 (4.41, 4.6)	
	Th3	4.83 (4.41, 4.6)	
	Th2	4.83 (4.41, 4.6)	
	Th4	6.25 (4.72, 4.85, 5.04)	6.2, 5.3 (De la Cruz *et al.* 1995a)
Chitinase	Th1	4.23-5.2, 6.1, 7.00-7.45	7.4 (Deane *et al.* 1998) 6.2 (De la Cruz *et al.* 1992)
	Th3	4.23-5.2, 7.00-7.45	
	Th2	7.23, 7.36, 7.83 (4.62-6.35)	7.8 and 4.6 (De la Cruz *et al.* 1992) 7.4 (Deane *et al.* 1998)
	Th4	7.23, 7.36, 7.83 (4.62-6.35)	as Th2
Protease	Th1	9.57, 5.53, 7.23	Pr1 and Pr2 (St. Leger *et al.* 1987)* Prb1 (Geremia *et al.* 1993)
	Th3	6.22, 9.57, 8.94-9.5	as Th1
	Th2	6.22, 9.57, 7.23	as Th1
	Th4	6.22, 9.57 (5.22, 5.53)	as Th1

* Pr1 and Pr2 are produced by *M. anisopliae*.

chitinase activity induced by crab shell chitin and revealed isoforms (pI 7.8 and 4.6) with pIs similar to Th2- and Th4-specific chitinase isoforms found here (pI 7.83 and 4.62). However, the two endochitinases found by De la Cruz *et al.* (1992) were induced by crab shell chitin and not by *B. cinerea* cell walls. Deane *et al.* (1998) reported an exochitinase induced by crab shell chitin, which had a pI comparable to a chitinase isoform (pI 7.36) in this work produced by Th2 and Th4 exposed to *A. bisporus* cell walls.

Not all chitinase isoforms may have dominant roles in mycoparasitism but synthesis may be triggered simultaneously with that of protease as suggested by the timing of production. The isoforms unique to aggressive strains may be more efficient at cell wall degradation and production was most rapid in these strains since more isoforms were produced (after 12 hours induction) than by Th1 and Th3. Lorito *et al.* (1998) reported that some endochitinases of *T. harzianum* are particularly potent in terms of antifungal activity and therefore this indicates that chitinase isoforms specific to aggressive isolates found here may be important for mycoparasitism. Chitinases may also be triggered by self-parasitism, behaviour common to *T. harzianum*, or to enable hyphal fusion to establish a network by which transport is more efficient (Peberdy 1990).

All genotypes displayed different protease isoforms, however there were also some isoforms unique to the aggressive genotypes (Table 4.1). Th2 and Th4 produced a dominant isoform (pI 6.22), which was also secreted by Th3 but was detected in substantially lower quantities. Detection of a protease isoform predominant to aggressive genotypes supports the rapid increase in Prl-like and Pr2-like activity specific to Th2 and Th4 (in this work) and suggests a possible role for this isoform in mycoparasitism. This theory is based on the importance of proteases in several other systems involving antagonism of a parasitic nature. These systems include: Prb1 secreted by *T. harzianum* in the presence of *R. solani* (Geremia *et al.* 1993; Flores *et al.* 1997), protease activity of *T. harzianum* when attacking *S. rolfsii* (Elad *et al.* 1982), Prl and Pr2 of the entomopathogen *M. anisopliae* (St. Leger *et al.* 1987a,b) and an alkaline trypsin-like protease secreted by the phytopathogen, *Stagonospora nodorum* (Carlile *et al.* 1999).

Th3 (European non-aggressive) exhibited quite different depolymerase isozyme profiles when compared with the other genotypes. The only exception was the laminarinase profile, which was possibly constitutive activity. Otherwise Th3 displayed isoform profiles with

similarities to the other genotypes as well as secreting many additional isoforms. Th3 demonstrated the ability to colonise compost to a greater extent than by the non-aggressive Th1 (see Chapters 3 and 5) and in contrast to other studies it also exhibited some aggressive behaviour towards *A. bisporus* (see Chapter 3). Th3 has recently been re-classified as *T. atroviride* (Muthumeenakshi *et al.* 1998). However for the duration of this work it was considered to be *T. harzianum* and a non-aggressive coloniser of mushroom compost.

This in depth study of extracellular depolymerase isoforms enabled the classification of *T. harzianum* strains according to the secretion of cell wall-degrading isozymes in response to exposure to potential host cell walls. A protease isoform with pI of 6.22 could possibly function in a mycoparasitic event directed towards *A. bisporus*. It must be made clear that such an antagonistic interaction may not be common and may only occur under specific conditions such as nutrient stress. However in comparison with *V. fungicola*, a recognised pathogen of the commercial mushroom, *T. harzianum* secretes a similar range of depolymerases. Both fungi secrete endo-β-1,3-glucanase, chitinase, and protease in the presence of *A. bisporus* cell walls (this work; Calonje *et al.* 1997).

Although genotype differences linked to aggressiveness were only detected for protease and chitinase activities, these differences may be enhanced by the synergistic action of the depolymerases that were secreted by all genotypes. Individually or in combination with other depolymerases, the isoforms linked with aggressiveness may be accountable for the aggressive behaviour of Th2 and Th4. Suggestions for future research aimed at evaluating the contributions of these enzymes are made in Chapter Six.

Higher depolymerase activities on exposure to *A. bisporus* cell walls compared to activities from exposure to chitin may indicate recognition of a potential host or merely different degrees of derepression according to the available carbon sources. The depolymerases necessary for host cell wall degradation were secreted by all genotypes, however there were some differences such as the rapid Pr1-like and Pr2-like activities of the aggressive genotypes. Different depolymerase isoforms were distinctive for genotypes and geographical origin. The APIZYM profiles revealed genotype differences from crude culture fluids. Genotypes were therefore distinguished at several levels pertaining to enzyme activities with possible roles in mycoparasitism towards *A. bisporus*.

Chapter Five

Saprophytic ability of *Trichoderma harzianum* genotypes on mushroom compost

5.0 Introduction

Trichoderma harzianum is commonly isolated from soil, where it exists as a saprotroph (Rifai 1969). This saprotroph is ubiquitous in most soils due to its highly competitive and combative nature; *T. harzianum* is capable of rapid colonisation, producing vast numbers of reproductive propagules and secretes an extensive range of polysaccharide depolymerases.

Many species of *Trichoderma* have been detected in association with the compost used in mushroom production (Fletcher 1986, Seaby 1996b). However these species, including *T. koningii* and *T.viride*, have had no apparent effects on crop yields. *Trichoderma* species may colonise compost or wooden trays in a localised manner, but only aggressive genotypes of *T. harzianum* are capable of destroying the complete mushroom crop. It has been suggested that green moulds, such as those associated with *Trichoderma* species, are an indication of insufficiently 'finished' compost (Fletcher *et al.* 1989).

The composition of mushroom compost is primarily, wheat straw. In order to colonise compost *T. harzianum* must obtain nutrients from straw by means of hemicellulases and cellulases. While the literature cites *T. harzianum* strains capable of producing and secreting cellulases and xylanases (Thrane *et al.* 1997; Campbell *et al.* 1993), most of the

research has been based on *T. reesei* (Teeri *et al.* 1998). The cellulolytic system of *T. reesei* is extensive and cellulases account for a large proportion of the proteins secreted (Goyal *et al.* 1991).

Crystalline cellulose is one of the most recalcitrant polysaccharide polymers. Limited genera of fungi produce enzymes capable of degrading native cellulose; these include the Basidiomycetes. Degradation of crystalline cellulose involves endo- and exo-acting enzymes. Endoglucanases cleave at random, internal sites while the cellobiohydrolases cleave cellobiose and glucose from ends of cellulose fibrils in a progressive action. Glucosidases cleave glucose monomers with exo-action from ends of cellulose fibrils in addition to splitting cellobiose.

Several species of *Trichoderma* can be detected in mushroom compost in addition to the four genotypes of *T. harzianum*. Efficient control will require effective and rapid identification of genotypes capable of aggressive colonisation. Therefore differences in biochemical and molecular characteristics must be accumulated. These techniques may not only yield early diagnosis but aid in the understanding of the mechanisms involved in interactions between *T. harzianum* and *A. bisporus*.

The aggressive, compost-colonising strains of *T. harzianum* may be apparent before or after spawning. In both situations *T. harzianum* is capable of surviving in compost and this may suggest a saprophytic mode of living before contact with the potential host, *A. bisporus*. Survival in mushroom compost or even on contaminated spawn grain presumably requires the secretion of hemicellulases and cellulases. Therefore this research was designed to investigate indirectly the saprophytic activity of *T. harzianum* in mushroom compost by determination of its relevant depolymerases. Quantification of *T. harzianum* growth in compost and the comparison of isozyme profiles provided an

indication of saprophytic abilities of genotypes. The experimentation addressed the possibility of initial saprophytic colonisation and its importance in the interaction between aggressive genotypes of *T. harzianum* and *A. bisporus*.

5.1 Results

5.1.1 Quantification of T. harzianum genotypes biomass in compost in the absence of A. bisporus

Quantification of the growth of *T. harzianum* genotypes in compost would allow a level of comparison of their relative saprophytic abilities. A reproducible method of quantification was required for determination of colony forming units (cfu). The recovery of *T. harzianum* cfu from non-sterile compost required a selective medium (Materials and Methods 2.1.2). Several media selective for *T. harzianum* have been described (Elad *et al.* 1981; Papavizas and Lumsden 1982; Smith *et al.* 1990). The medium chosen was an adaptation of a medium developed by Askew and Laing (1993). The recovery of cfu from compost samples was adapted from Cazemier and coworkers (1997) (see Materials and Methods 2.1.2).

5.1.1(i) Development of a selective medium for T.harzianum

The process of selective medium development included several variations on the medium described by Askew and Laing (1993). Initially combinations were made using 2% w/v malt extract agar (MEA) supplemented with combinations of antibiotics (chloramphenicol, streptomycin sulphate and nystatin, Sigma, UK) to prevent growth of bacteria and actinomycetes present in compost, fungicides (quintozene, Sigma, UK; captan, kindly donated by Zeneca Agrochemicals, UK; propamocarb hydrochloride, kindly donated by Levington Horticulture, UK) and sodium deoxycholate (Sigma, UK). Various media were compared for their effects on *T. harzianum* growth and germination characteristics (Table 5.1). The eventual selective medium supported the growth of *T. harzianum* alone and this is significant as mushroom compost harbours a substantial microflora. Selective medium was also inoculated with extracts from non-sterile compost to which *T. harzianum* spores had not been added and after incubation these plates were clean (Fig 5.1).

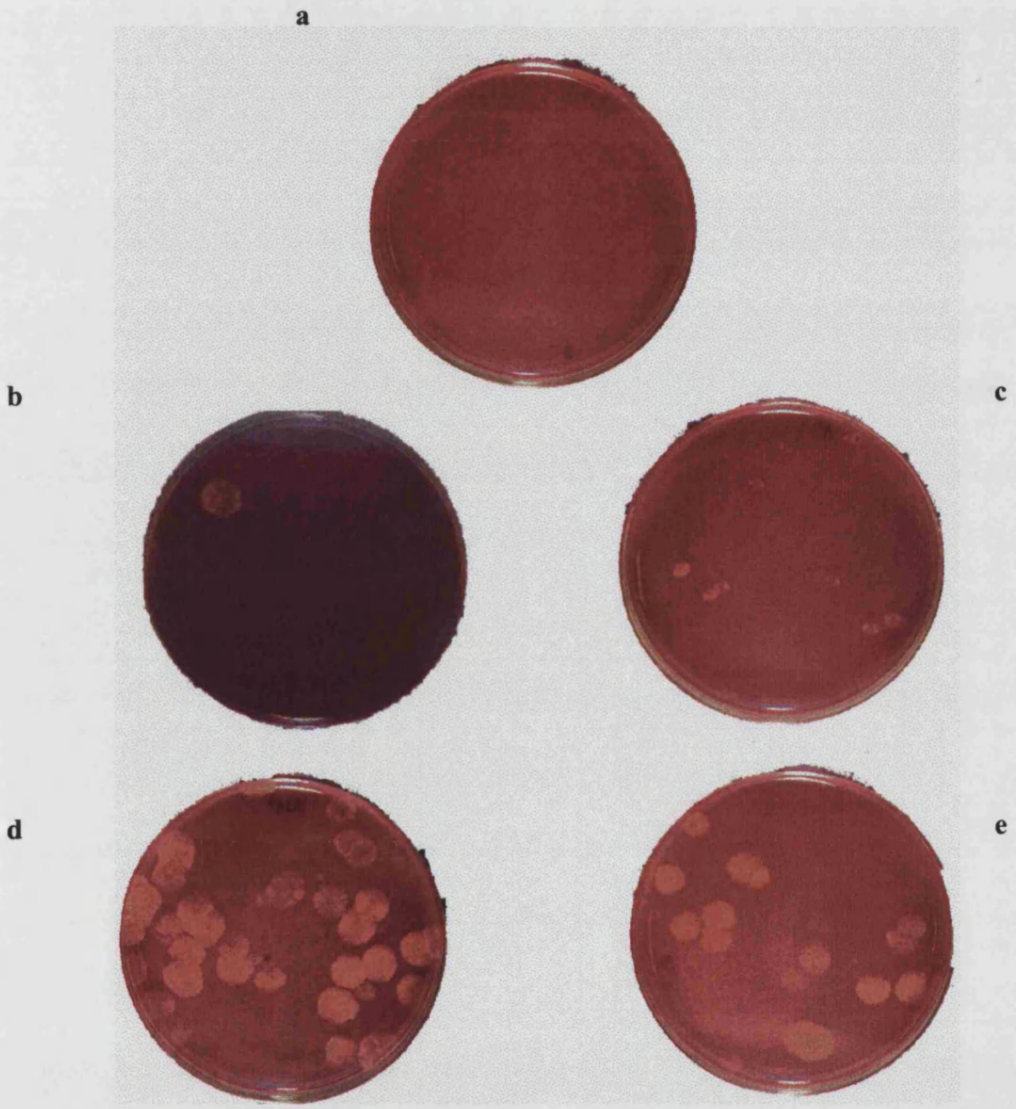
Media were inoculated with mycelial plugs or 100µl of spore suspensions (10^3, 10^2, 10, 10^{-1} spores ml^{-1}) of *T. harzianum* strains T7 (Th2) and Th1(c) (Th1). Plates were incubated at 25°C in the absence of light and daily assessments were made of colony diameter. Non-aggressive and aggressive strains of *T. harzianum* were assessed for growth on selective media to ensure that both were capable of colonising the media and prevent false interpretations of the presence/absence of *T. harzianum*. This also allowed

Table 5.1: Investigation of effect on growth and germination of *T. harzianum* of various selective.

Medium Name	Medium components
malt extract agar	2% w/v MEA
medium 1	2% MEA, chloramphenicol, streptomycin, sodium deoxycholate
medium 2	2% MEA, chloramphenicol, streptomycin
medium 3	2% MEA, chloramphenicol, streptomycin, nystatin
medium 4	2% MEA, chloramphenicol, streptomycin, captan, propamocarb
Trichoderma selective medium (TSM)	see Materials and Methods 2.1.2

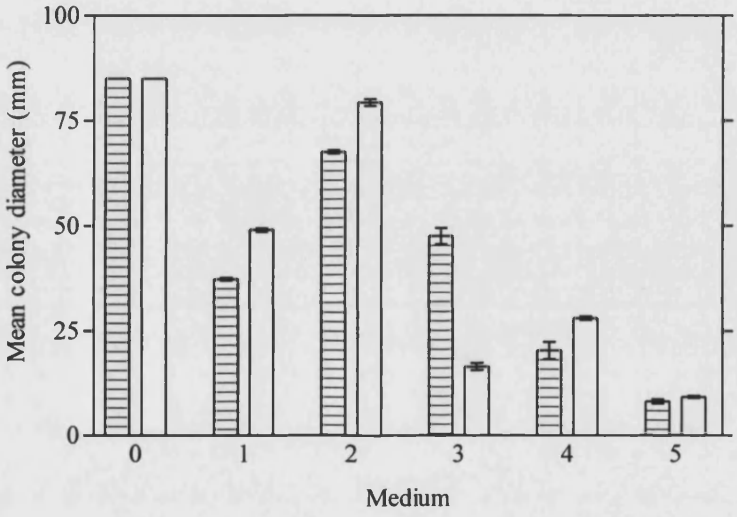
MEA: malt extract agar.

Figure 5.1: Comparison of cfu from saprophytic growth of *T. harzianum* genotypes detected on *Trichoderma* selective medium.



(a) non-sterile compost control, (b) strain Th1(c) (Th1), (c) A006022 (Th3), (d) Th2A (Th2) and (e) RM10c (Th4). The plates shown were diluted 10^{-3} and are representative of a $\log_{10}$ dilution series.

Figure 5.2: Hyphal growth of *T. harzianum* strains on various selective media.



All measurements taken after 3 days incubation with the exception of medium 3 which was taken on day 12.

Numbers on X axis represent: 1-4: media 1-4; 0: MEA; 5: TSM.

Bars indicate standard error of the mean (five replicates).

T. harzianum isolate: Th1(c)(Th1) T7 (Th2)

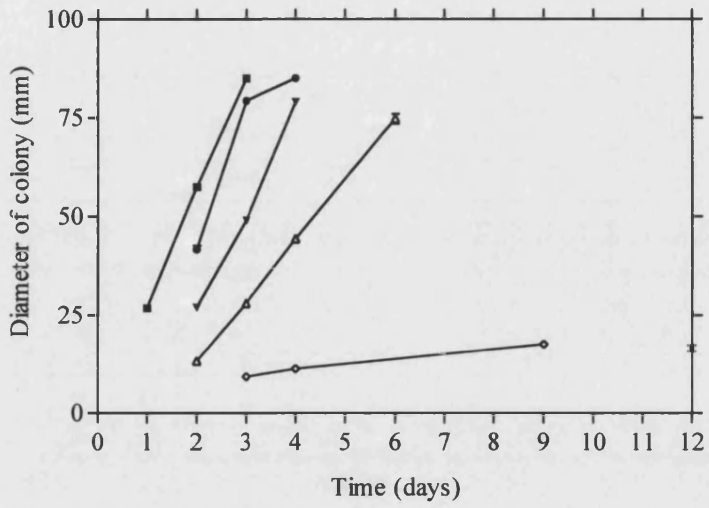
determination of any unusual growth characteristics that may be observed between the two genotypes.

T. harzianum strain Th1(c) (Th1) and T7 (Th2) appeared to have identical growth rates on MEA (Table 5.2). The inclusion of antibiotics and the growth inhibitor, sodium deoxycholate (medium 1 – Table 5.1), had an inhibitory effect on both strains (*ca.* 1.12-fold and 1.43-fold, for Th1(c) and T7 respectively). The addition of antibiotics alone had an apparent stimulatory effect, *ca.* 1.30-fold and 1.07-fold for strain Th1(c) and T7 respectively. Nystatin, an antifungal compound, substantially inhibited the growth of both *T. harzianum* strains and the growth rate of strain Th1(c) could not be calculated as growth was detected the last data collection time only. *T. harzianum* strain T7 exhibited a 4.32-fold decrease in growth rate in the presence of nystatin (medium 3). The addition of fungicides to MEA (medium 4) had inhibitory effects on *T. harzianum* growth, displayed typically as *ca.* 1.89-fold and 1.59-fold decrease in growth rates (Table 5.2). Growth rates of *T. harzianum* strains on TSM also exhibited an inhibitory effect and the reduction in rate was *ca.* 14.58-fold and 2.95-fold for Th1(c) and T7 respectively.

Growth on all media was substantially reduced (at least 2-fold to 10-fold depending on the medium) when compared to the MEA control (Table 5.3). Colony diameters of strains T7 and Th1(c) were not significantly different, except on medium 3, on which the diameter of *T. harzianum* strain T7, on day 12, was significantly higher than Th1(c) (Table 5.3).

Growth on malt extract agar (MEA) and media 1, 2 and 4 revealed little difference in colony diameter between strains of *T. harzianum*. Colony growth from mycelial plugs on MEA was detected after 24 hours for both *T. harzianum* strains, while colony growth on media 1, 2 and 4 was not detected until day 2 (Fig 5.3). Growth on TSM was delayed and not observed until day 3 for both T7 and Th1(c). Although both strains had similar colony diameters on day 3 (Table 5.3) continued growth of T7 colony was substantially higher than Th1(c) (x3.5-fold) (Fig 5.3).

Similarly, differences were seen in the growth of T7 and Th1(c) on medium 3, which differed from the other media by the inclusion of nystatin. Colony growth of *T. harzianum*



a

b

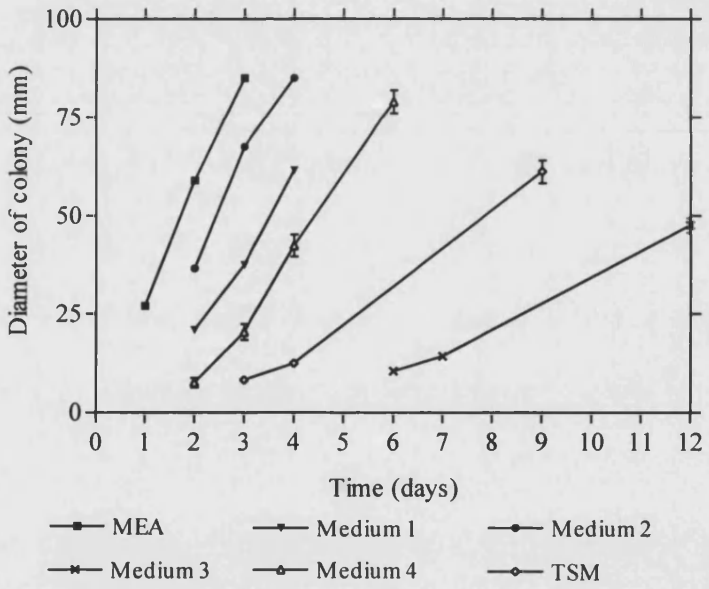


Figure 5.3: Temporal measurements of *T. harzianum* hyphal growth on various media.
a) *T. harzianum* strain Th1(c) (Th1). b) *T. harzianum* strain T7 (Th2).
Bars indicate standard error of the mean.

Table 5.2: Growth rates of *T. harzianum* strains on various selective media.

Medium*	Growth rate of strains of *T. harzianum* (mm hr^{-1})	
	Th1(c)	T7
MEA	1.21	1.21
1	1.08	0.85
2	1.57	1.29
3	nd	0.28
4	0.64	0.76
TSM	0.083	0.41

* Media used – see explanatory notes on Table 5.1.

nd – rate of growth could not be determined for strain Th1(c) on medium 3 as growth measurements were taken over 12 days and growth of Th1(c) was first detected on day 12.

Genotype 1: Th1(c), genotype 2: T7.

Table 5.3: Germination and hyphal growth of *T. harzianum* strains on various media after 3 days incubation

Medium	Germination	Diameter of colonies* (mm) Th1(c)	T7
MEA	Yes	85[a]	85[a]
1	Yes	49	37
2	Yes	79 (± 1)	68
3	No	17[Ψ] (± 1)	48[Ψ] (± 2)
4	No	28	20 (± 2)
TSM	No	9	8 (± 1)

* Mean diameter of colony growth from mycelial plugs. Standard error of the mean is given in parentheses. [Ψ] Colony growth was only detected at day 12 on medium 3; all other measurements were recorded on day 3. Colony diameters on medium 3 are significantly different when analysed using a two-tailed paired t-test with 95% confidence levels.

[a] Colony diameters of both *T. harzianum* strains on media 1-4 and TSM are significantly different when compared with MEA controls by analysis with two-tailed paired t-test, with 95% confidence levels.

Table 5.4: The effect of fungicides on germination of *T. harzianum* spores.

Fungicide	Spore number (ml^{-1})	Mean number of colonies	Mean diameter of colonies (mm)
Propamocarb	10^3	**	**
	10^2	11 (± 1.155)*	10 ± 1
	10^1	2.67 (± 1.453)	10
	10^{-1}	0	
Quintozene	10^3	92.33 (± 4.256)	4 ± 1
	10^2	9.33 (± 1.667)	4
	10^1	1.67 (± 1.667)	4
	10^{-1}	0.33 (± 0.333)	4
Rose Bengal	10^3	124.67 (± 10.171)	2 ± 1
	10^2	13.67 (± 0.333)	2
	10^1	4.33 (± 0.667)	2
	10^{-1}	0.33 (± 0.333)	2
Q and P	10^3	**	**
	10^2	13 (± 0.577)	6 ± 1
	10^1	1.33 (± 0.333)	6
	10^{-1}	1 (± 0.577)	6

* Standard error of the mean in parentheses (of five replicates). Q – Quintozene and P – Propamocarb. ** Too many colonies to assess.

strain T7 was not detected on medium 3 until day 6, after which it continued to grow but not to the same extent as on other media. Strain Th1(c) failed to establish growth on medium 3 from mycelial plugs until day 12 (Fig 5.2a). Strain T7 colony growth was 2.88-fold higher than Th1(c) after 12 days incubation on medium 3 (Fig 5.3).

Medium 1, which contained sodium deoxycholate, inhibited growth of both strains of *T. harzianum* more than medium 2; an identical medium without sodium deoxycholate. Medium 4, similar to TSM in antimicrobial ingredients, inhibited growth of both Th1(c) and T7 compared to MEA and media 1 and 2 (Fig 5.3).

Germination only occurred on MEA and media 1 and 2 (Table 5.3). Media 3, 4 and TSM all inhibited germination of both strains of *T. harzianum*. Therefore the individual fungicidal ingredients of TSM were investigated for ability to prevent the germination of *T. harzianum*. The fungicidal components of TSM were captan, propamocarb-hydrochloride, quintozene and the growth inhibitor rose bengal. These ingredients were added individually to MEA and in the following combinations: i) quintozene and propamocarb, ii) quintozene and captan and iii) propamocarb and captan. Media were inoculated with a dilution series (10^3, 10^2, 10, 10^{-1} spores ml^{-1}) of *T. harzianum* strain T7 spores and incubated at 25°C.

Spores of *T. harzianum* strain T7 germinated successfully on all fungicidal components except captan and any combination including captan. Rose Bengal inhibited colony growth compared to propamocarb and quintozene and combinations of the two (Table 5.4). The cfu were as expected according to the dilutions inoculated. In view of these data the final selective medium was identical to that of Askew and Laing (1993) with the exceptions of the addition of streptomycin sulfate (Sigma, UK) and the absence of captan. The selectivity of the medium becomes apparent from reisolation of *T. harzianum* from non-sterile compost below.

5.1.1(ii) Comparison of saprophytic growth in compost of aggressive and non-aggressive genotypes of T.harzianum

At least two and in most instances, three strains from each genotype were investigated for their saprophytic growth capabilities. Spores of *T. harzianum* were introduced to non-sterile compost on sterile rye grain and cfu were recovered after 3 weeks incubation at

25°C with high relative humidity of *ca.* 80% (see Materials and Methods 2.1.2). It was not possible to coat the rye grain with a pre-determined number of *T. harzianum* spores and therefore cfu were quantified from a sample of 10 grains coated with spores and the results compared. Colony forming units from the sample of grains was termed the inoculum, while recovered cfu were indicative of saprophytic growth.

Agitation of the sample of ten grains in 10 ml of sodium tetrapyrophosphate [100 mM] for 2 minutes in a Stomacher Lab-Blender 400 and subsequent filtration through muslin, released the inoculum cfu. Quantification of inoculum spore load was performed as an internal control to ensure that any differences in saprophytic growth were not as a consequence of possible variation in inocula. The inocula for ten strains showed no significant difference and should not be compared with saprophytic growth; it is simply included for validity. Colony forming units were also recovered from a 10 g sample of colonised compost in 100 mM sodium tetrapyrophosphate (Cazemier *et al.* 1997). The compost was steeped in 100 ml of sodium tetrapyrophosphate [100 mM] for 1 hour at room temperature, prior to continuous agitation in a Stomacher Lab-Blender 400 for 2 minutes. Aliquots of 1 ml were taken from both inoculum and from samples for saprophytic growth and diluted (1:10) to give a log dilution series. Selective medium (TSM) plates were inoculated with 100 µl of each dilution and incubated at 25°C until discrete colonies were visible. Colonies were quantified using a Stuart Scientific colony counter.

To analyse the data the colony counts were $\log_{10}$ transformed, treated as geometrical with a normal distribution and subjected to one-way analysis of variance (ANOVA) using Minitab for windows. The results were compared with analysis of the original counts, which had Poisson distribution and were analysed using Genstat. The two methods gave comparable results and therefore ANOVA was chosen for convenience.

Statistical analysis of inoculum colony counts by ANOVA revealed no significant differences between the strains and therefore this allows the direct comparison of saprophytic growth of each strain with no significant biased effects from unbalanced quantities of inoculum (Fig 5.4).

Strains of *T. harzianum* varied in growth capabilities in non-sterile compost and these capabilities ranged from 3.0 to 5.11 cfu (log_{10}) (Fig 5.4 and Table 5.5). Genotype 1 strains (Th1(c), TD15 and T28JF) exhibited reduced ability, typically 3.0 to 4.34 cfu (log_{10}), to colonise non-sterile compost and cfu counts were significantly lower than those of genotypes 2, 3 and 4, which exhibited cfu in the range 4.37 to 5.11 (log_{10}). Saprophytic growth of strains from genotypes 2, 3 and 4 were very similar; some slight differences were observed, however these were of far lower magnitude than the differences between these three genotypes and genotype 1.

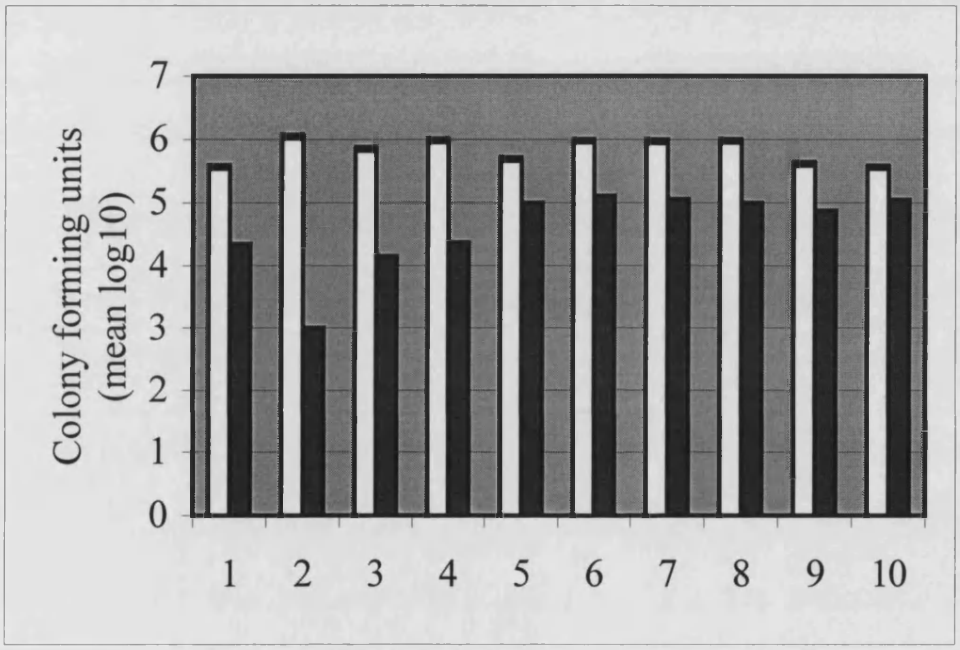
5.1.2 Production of cereal cell wall depolymerising enzymes by T. harzianum genotypes in vitro in the presence of wheat straw

In the absence of resting structures survival within non-sterile mushroom compost in the absence of *A. bisporus* would require saprophytic growth by *T. harzianum*. The rye grain on which spores of *T. harzianum* were inoculated in the model would provide an initial source of nutrients, mainly as hemicelluloses (Kent 1983). Successful colonisation of mushroom compost may be achieved if the cellulose and hemicellulose content of wheat straw component was degraded. Several strains from each genotype of *T. harzianum* were investigated for secretion of cellulase and hemicellulase. *T. harzianum* was cultured *in vitro* in the presence of chopped wheat straw (1% w/v) and culture fluids were assayed for enzyme activity.

Spore suspensions (1 ml of 10^7 spores ml^{-1}) of *T. harzianum* were used to inoculate 100 ml of basal medium (Cooper and Wood 1975) supplemented with 1% w/v glucose to produce sufficient mycelial growth (*ca.* 500 mg dry weight) after three days. The mycelia were then washed and starved overnight in 100 ml fresh basal medium and subsequently transferred to basal medium supplemented with 1% w/v wheat straw. Flasks were returned to incubator at 25°C with shaking at 180 rpm, in the absence of light. Samples of culture fluids were removed at specific time points and clarified by centrifugation.

A range of conditions was employed to characterise depolymerases of *T. harzianum* capable of degrading wheat straw. Optimal pH for activity was determined over a pH range which spanned 4.0 to 11.0 in half units, using five different 50mM buffer systems

Figure 5.4: Quantification of saprophytic growth of *T. harzianum* isolates in non-sterile compost.



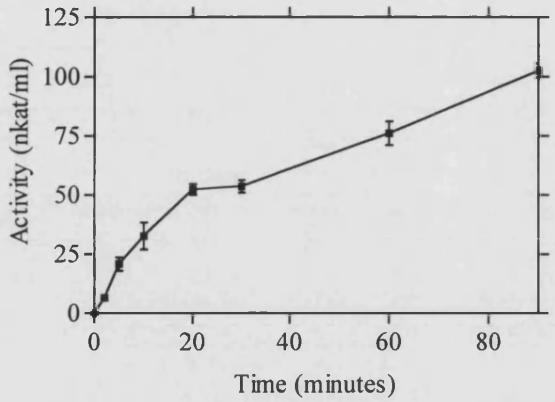
Figures given on x axis denote: 1, Th1(c); 2, TD15; 3, T28JF; 4, T7; 5, Th2A; 6, KPNT; 7, TD7; 8, A006022; 9, RM10c; 10, BE. Bars indicate overall confidence limits for all strains. Confidence limits for inoculum are ± 0.0285 and for saprophytic growth the limits are ± 0.002.

Inoculum cfu Saprophytic growth cfu

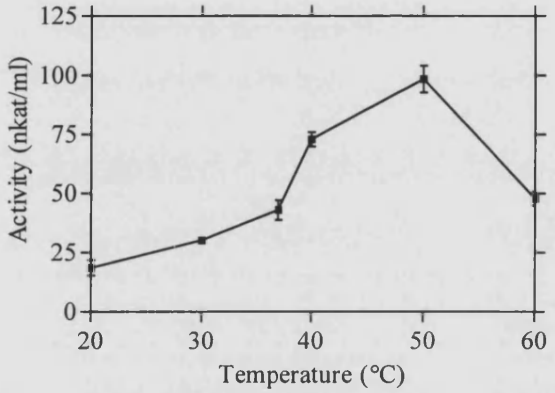
Table 5.5: Quantification and comparison of saprophytic growth of *T. harzianum* genotypes in non-sterile compost.

T. harzianum strain	Genotype	mean number of colony forming units (log_{10})
Th1(c)	Th1	4.34[a]
TD15	Th1	3.00[b]
T28JF	Th1	4.15[c]
T7	Th2	4.37[a]
Th2A	Th2	5.00[d, f, g]
KPNT	Th2	5.11[d, e]
TD7	Th3	5.06[d, e, f]
A006022	Th3	5.00[f]
RM10casing	Th4	4.87[g]
BE	Th4	5.05[d, f]

[a] cfu's followed by different letters are significantly different ($P \leq 0.05$) according to one-way ANOVA and Fisher's multiple comparison test.



a



b

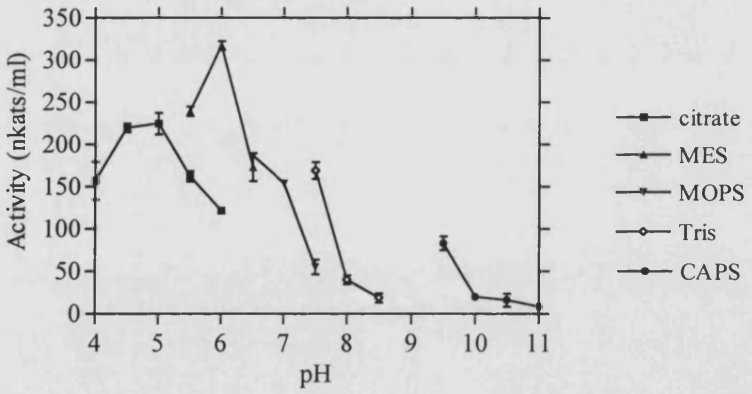


Figure 5.5: Optimal conditions of a) incubation time, b) temperature and c) pH for cellulase assays. Bars indicate standard error of the mean with three replicates. All activities calculated as nkat/ml/g dry weight mycelium.

which each displayed buffering capacities over a certain range and several showed considerable overlap. Citrate buffer covered the range pH 4.0 to 6.0;MES buffered at pH 5.5 to 6.5; MOPS, pH 6.5 to 7.5; Tris-HCl buffer, pH 7.5 to 8.5 and CAPS, pH 9.5 to 11.0.

An investigation of optimal temperature for cellulase and xylanase activity used temperatures between 20 to 60°C. Incubation times within the range 2 to 120 minutes were chosen to study the optimal assay incubation time.

5.1.2(i) Characterisation of cellulases secreted by genotypes of T. harzianum in the presence of wheat straw

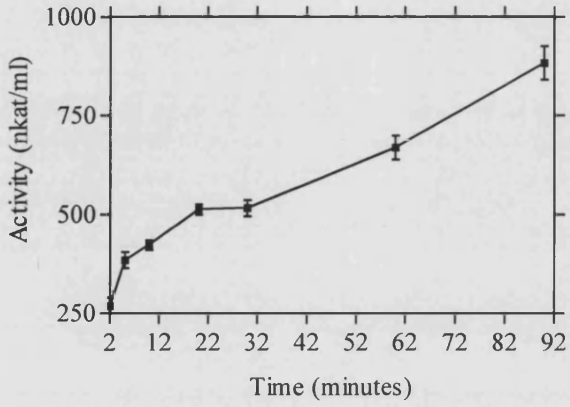
Cellulase activity exhibited a linear increase between 0 and 20 minutes. Extension of the incubation period beyond 20 minutes revealed a reduced and non-linear rate of reaction (Fig 5.5a). An optimal time of 20 minutes was chosen.

Temperatures between 20 and 37°C doubled cellulase activity and beyond 37°C there was a rapid increase in the rate of activity up to maximal temperature of 50°C. Beyond 50°C activity was greatly reduced (Fig 5.5b). An optimal incubation temperature of 50°C was determined after 20 minutes incubation. Different buffers [50 mM] revealed marked differences in cellulase activity and highlighted the importance of considering more than one buffer system (Fig 5.5c). High activities were recorded over pH range 4.0 to 7.5 with citrate and MES buffer systems. The extreme ranges of each buffer system revealed reduced activity and cellulase activity was substantially reduced at pH levels in excess of 7.5. Maximal cellulase activity occurred at pH 6.0 in 50 mM MES and this was chosen as the routine assay buffer. Citrate buffer at pH 6.0 exhibited substantially lower (*ca.* 2.6-fold) cellulase activity.

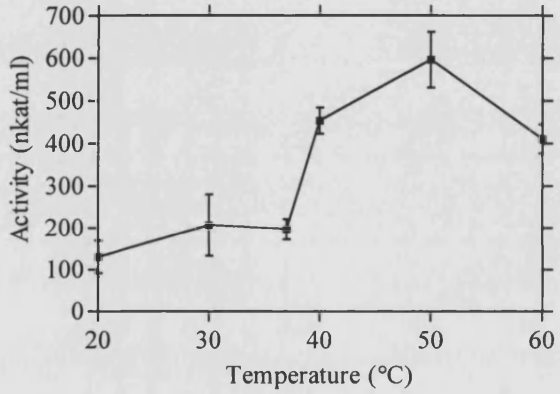
5.1.2(ii) Characterisation of xylanases secreted by genotypes of T. harzianum in the presence of wheat straw

Increase in xylanase activity between 0 and 20 minutes, while not initially linear then exhibited a rapid linear rate of reaction until 20 minutes, when rate of reaction was reduced (Fig 5.6a). An optimal incubation time of 20 minutes was chosen.

a



b



c

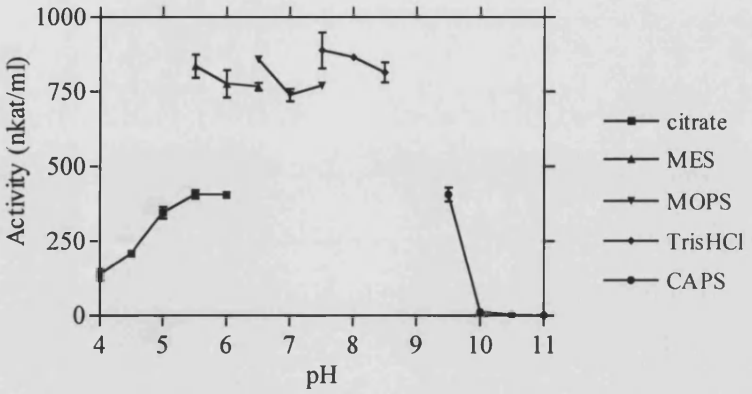
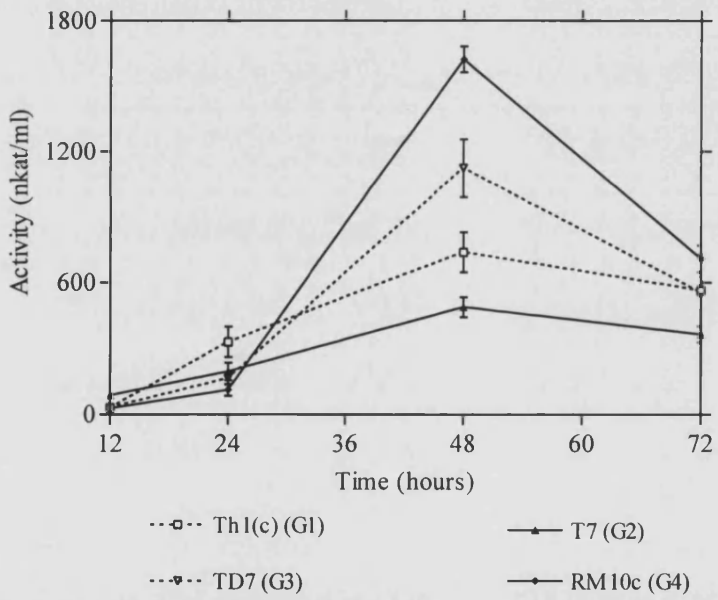


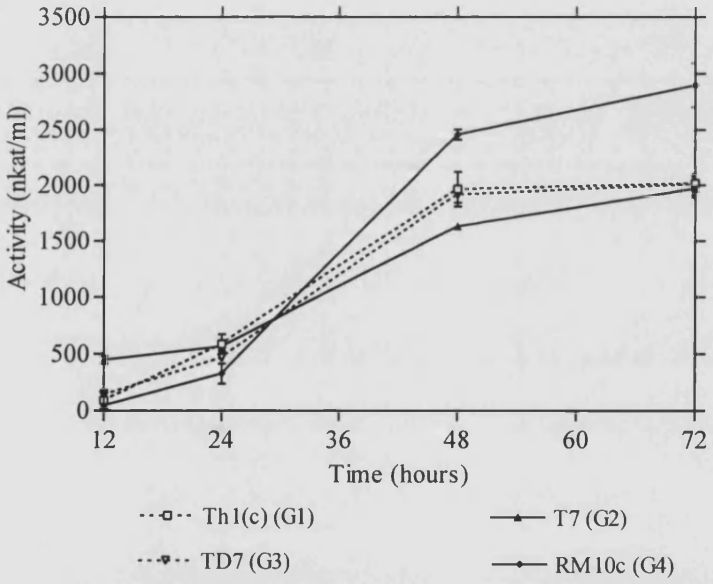
Figure 5.6: Optimal conditions of a) incubation time, b) temperature and c) pH for xylanase assays. Bars indicate standard error of the mean of three replicates. All activities calculated as nkat/mg/g dry weight mycelium.

Figure 5.7: Comparison of temporal production of cellulase by *T. harzianum* genotypes.



Bars indicate standard error of the mean of three replicates. Activity calculated as nkat/ml/g dry weight of mycelium.

Figure 5.8: Comparison of temporal production of xylanase by *T. harzianum* genotypes.



Bars indicate standard error of the mean of three replicates. Activity calculated as nkat/ml/g dry weight of mycelium.

Temperatures between 20 and 37°C had no marked effect on xylanase activity, beyond this range however, a sharp increase in activity was detected (Fig 5.6b). Maximal activity was recorded at 50°C above which there was a reduced rate of reaction. The optimal temperature was determined as 50°C.

Xylanase activity exhibited marked differences across the broad pH range (Fig 5.6c). Maximum activity occurred across the pH range 5.5 to 8.5 with MES, MOPS and Tris-HCl buffer systems. Tris-HCl at pH 7.5 provided maximal activity however, as there was little difference to the activity recorded using MES at pH 5.5, the latter was chosen as optimal for convenience. MES was also the buffer system used to stabilize culture medium as well as for all other enzyme assays.

5.1.2(iii) Comparison of temporal production of endo-β-1,4-glucanase by T. harzianum genotypes

Temporal production of one component of cellulase (endo-β-1,4-glucanase) in the presence of wheat straw was followed by the removal of culture fluid samples after 12, 24, 48 and 72 hours of induction. All genotypes secreted cellulase following a lag phase in the first 24 hours of induction (Fig 5.7). After 24 hours, genotypes 3 (TD7) and 4 (RM10c) displayed a marked and rapid increase in activity. Genotypes 1 (Th1(c)) and 2 (T7) also exhibited an increase in activity but at lower rate. Maximal activity was recorded after 48 hours induction for all four genotypes.

5.1.2(iv) Comparison of temporal production of endo-β-1,4-xylanase by T. harzianum genotypes

All genotypes produced extracellular xylanase activity. Genotype 2 (T7) demonstrated high xylanase activity during the first 24 hours of induction, when compared to the others (Fig 5.8). Between 24 and 48 hours, all genotypes showed a substantial increase in xylanase activity, which then declined in rate beyond 48 hours. The genotypes exhibited similar shape plots but differed in levels of activity. After 24 hours induction, genotype 4 (RM10c) exhibited the highest xylanase activity followed by genotypes 1 (Th1(c)) and 3 (TD7) and finally, genotype 2 (T7) which had the lowest activity.

5.1.3 Depolymerase isoforms produced by T. harzianum genotypes in vitro in the presence of wheat straw

Several isoforms of depolymerases may be produced. Separation of isoforms by iso-electric focussing may reveal a profile of isozymes specific to a particular genotype of *T. harzianum* and therefore the technique could be capable of identifying aggressive strains *T. harzianum*. Genotypic differences may provide an explanation or understanding of the antagonism employed by aggressive *T. harzianum* strains against *A. bisporus*. These experiments investigated the isozymes of depolymerases produced in the presence of wheat straw and during a possible an initial saprophytic stage, which may result in competition or antagonism directed towards *A. bisporus*. Isoforms of cellulase and xylanase were separated according to iso-electric points in free solution using Preparative Rotofor™ IEF and by gel electrophoresis using 5% Ampholine PAG plate gels (Pharmacia Biotech).

5.1.3(i) Isoform profiles of cellulase and xylanase activity produced by genotypes of T.harzianum separated according to isoelectric points in free solution

A culture fluid sample prepared from cultures supplemented with wheat straw was subjected to a pH gradient in a Preparative Rotofor™ IEF cell. Proteins were separated into 20 fractions according to iso-electric points (pI) (see Materials and Methods 2.3.2). The fractions were harvested, pH and protein content determined and enzyme activity assayed. Cellulase and xylanase activity was determined for each of four genotypes. The majority of cellulase activity for all four genotypes was recorded within the pH range 2.5 to 7.5 (Fig 5.9). Genotype 3 (TD7) exhibited the highest cellulase activity, typically *ca.* 3.33-fold higher than other genotypes. Little or no cellulases above pI 7.5 were detected.

Xylanase activities were observed across a broader pH range, 3.5 to 9.5 (Fig 5.10). The aggressive genotypes (2 and 4) demonstrated higher xylanase activity than genotype 1 (Th1(c)) and 3 (TD7), typically *ca.* 3.75-fold and 1.4-fold respectively. The fractions containing high xylanase activity for genotypes 2 and 4 were in the pH range 6.5 to 9.5. Genotypes 1 and 3 exhibited xylanase activity across a broad pH range, though activity gradually increased with an increase in pH.

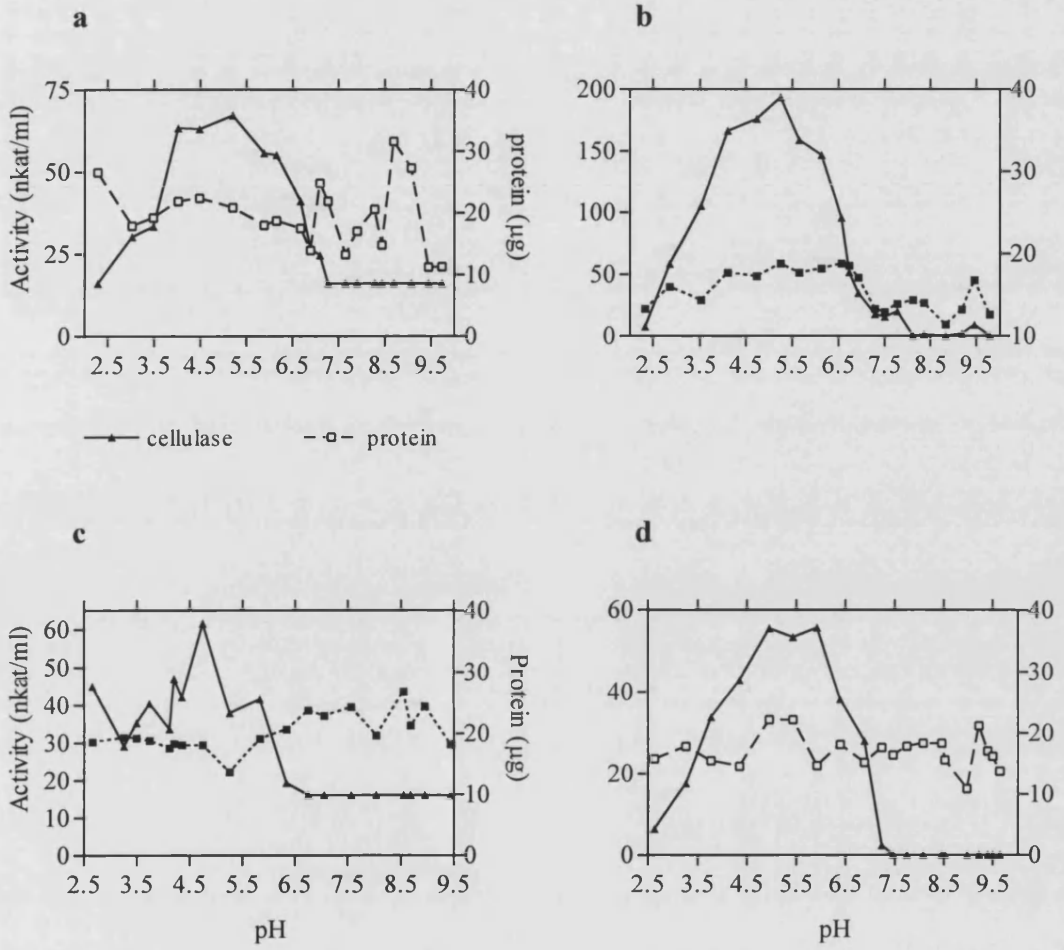


Figure 5.9: Activity of cellulase isoforms produced by *T. harzianum* genotypes in the presence of wheat straw. Preparative Rotofor™ used for iso-electric focusing.

a) strain Th1(c) (G1), b) strain TD7 (G3), c) T7 (G2) and d) RM10c (G4).

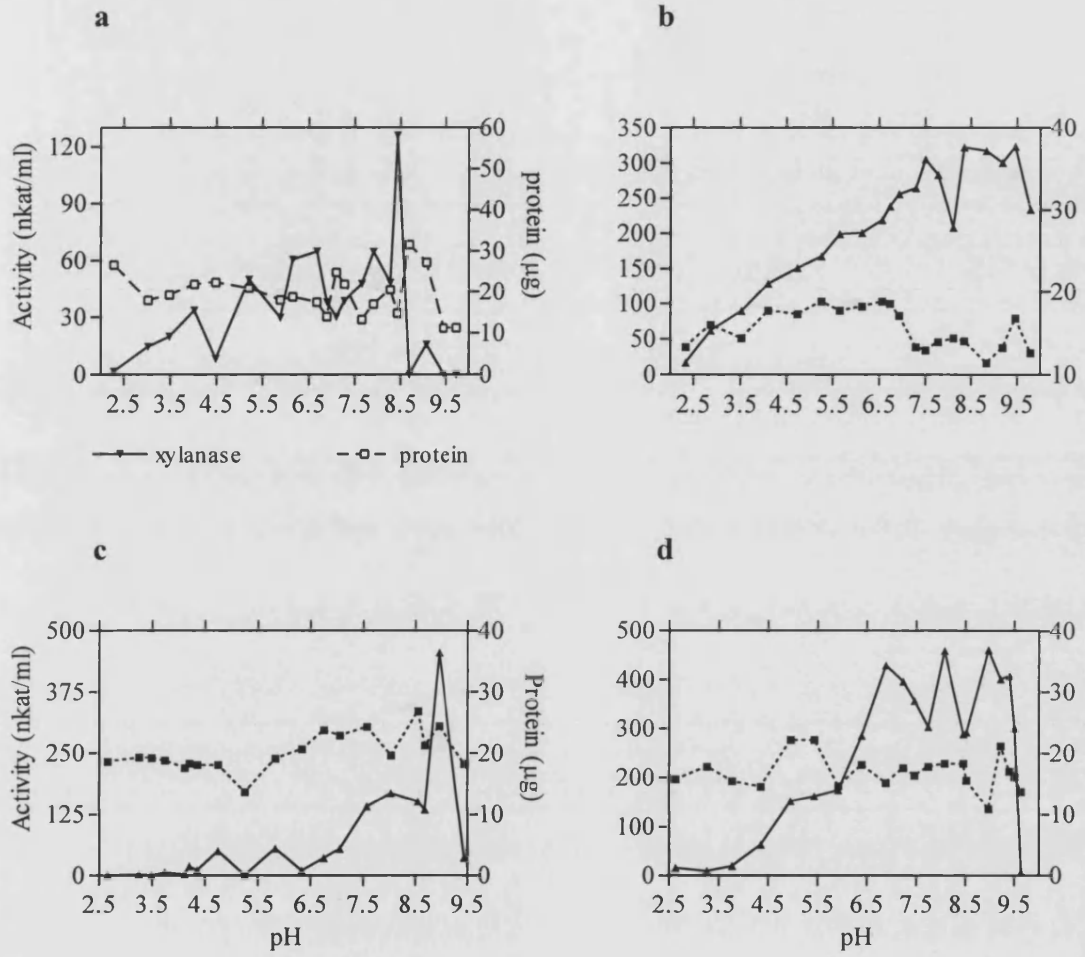


Figure 5.10: Activity of xylanase isoforms produced by *T. harzianum* genotypes in the presence of wheat straw. Preparative Rotofor™ for focus iso-electric focusing.

a) strain Th1(c) (G1), b) strain TD7 (G3), c) T7 (G2) and d) RM10c (G4).

5.1.3(ii) Isoform profiles of depolymerases secreted by genotypes of T.harzianum separated according to isoelectric points using gel electrophoresis

Iso-electric focussing was performed on samples of proteins removed after 12 and 24 hours induction from cultures supplemented with wheat straw. The samples were concentrated by lyophilisation (see Materials and Methods 2.3.3). Gels were prepared and samples were loaded according to manufacturer's instructions (see Materials and Methods 2.3.3)

5.1.3(iii) Isoform profiles of cellulases produced by T. harzianum genotypes in the presence of wheat straw

Cellulase activity was detected using a range of substrates dependent on the lytic action of the cellulase. Ostazin brilliant red-hydroxyethylcellulose (OBR-HEC) (Sigma, UK) was used to detect endo-β-1,4-glucanase and 4-methylumbelliferyl-β-D-cellobioside (Sigma, UK) distinguished cellobiohydrolase from β-glucosidase. All three classes of cellulase are capable of degrading 4-methylumbelliferyl-β-D-cellobioside (MUC) however a process of elimination may be employed to identify specific cellulases. Exocellobiohydrolases can degrade cellooligosaccharides (e.g. MUC), but not OBR-HEC. In this work β-glucosidases were inhibited from MUC degradation by 10 mM gluconolactone (Coughlan 1988; Todorovic *et al.* 1990).

All genotypes produced a complex array of cellulase isoforms (Fig 5.11). Isoforms of endo-β-1,4-glucanase, β-glucosidase and cellobiohydrolase were apparent for all four genotypes.

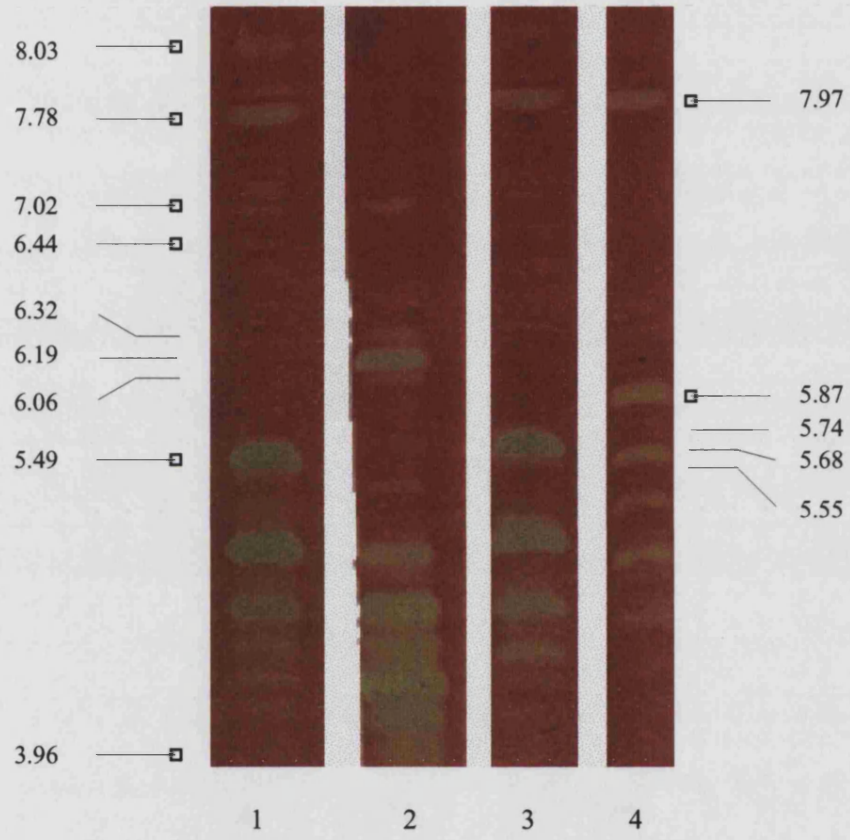
After 12 hours induction in culture isoforms of endo-β-1,4-glucanase and β-glucosidase were detected from non-aggressive genotypes (1 and 3) and aggressive genotype 2 (Fig 5.11 and 5.12a). Genotype 1 produced one isoform with pI 7.46 and genotype 2 produced several endo-β-1,4-glucanase isoforms with a broad range of iso-electric points (pI): 4.72 to 7.59. Genotype 3 also displayed several endo-β-1,4-glucanase isoforms, however the pI range was more acidic than genotype 2: pI 4.02 to 5.23. Only genotypes 1, 2 and 3 exhibited β-glucosidase activity. Genotypes 1 and 2 produced an identical β-glucosidase isoform with a pI of 8.61 after 12 hours induction. In addition the non-aggressive genotypes (1 and 3) produced β-glucosidase isoforms with pIs of 6.38 and 6.70 and 6.25 and 6.51, respectively. No cellobiohydrolase activity was detected after 12 hours induction.

Induction for 24 hours resulted in the production of many isoforms of endo-β-1,4-glucanase for all genotypes (Fig 5.11). Endo-β-1,4-glucanase isoforms produced by genotypes 1 (non-aggressive), 2 and 4 (aggressive) were clustered into two groups: those with alkaline pI and those with acidic. Genotype 3 exhibited endo-β-1,4-glucanase isozymes with a broad range of pIs (pH 3.96 to 8.29). The aggressive genotypes (2 and 4) appeared to secrete a common endo-β-1,4-glucanase with pI 7.97. *T. harzianum* strain Th1(c) (Th1) exhibited more alkaline isoforms of endo-β-1,4-glucanase than any other genotype. In addition aggressive genotypes (2 and 4) and genotype 3 (non-aggressive) produced several isoforms not demonstrated by genotype 1. These isoforms of endo-β-1,4-glucanase had pI's of 5.55 and 5.74 (T7 (Th2)), 5.68 and 5.87 (RM10c (Th4)) and 5.68, 6.06, 6.19 and 6.32 (TD7 (Th3)).

Isozymes of β-glucosidase, secreted after 24 hours induction, appeared to have very similar pI's to those detected after 12 hours induction. Genotypes 1 and 2 continued to secrete a β-glucosidase isoform with pI 8.61 (Fig 5.12b). In addition, isoforms with pI 6.00 and 7.97 were detected from genotype 1 (Th1(c)). Genotype 3 (TD7) appeared to secrete β-glucosidase isoforms with pI 6.19 and 7.46. An isoform with pI 8.29 was produced by genotype 4 (BE) after 24 hours induction.

Cellobiohydrolase activity was only detected after 24 hours induction, for all genotypes (Fig 5.12b). Isoforms with pI 5.36 and 9.24 were common to genotypes 1 (non- aggressive) and 2 and 4 (aggressive). Genotype 3 (non-aggressive) produced two apparent isoforms of cellobiohydrolase with pI 4.15 and 4.72. Genotype 1 secreted an additional isoform with pI 4.85.

Figure 5.11: Isoforms of endo-β-1,4-glucanase produced by *T. harzianum* genotypes in the presence of wheat straw.



After 24 hours induction. Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4).

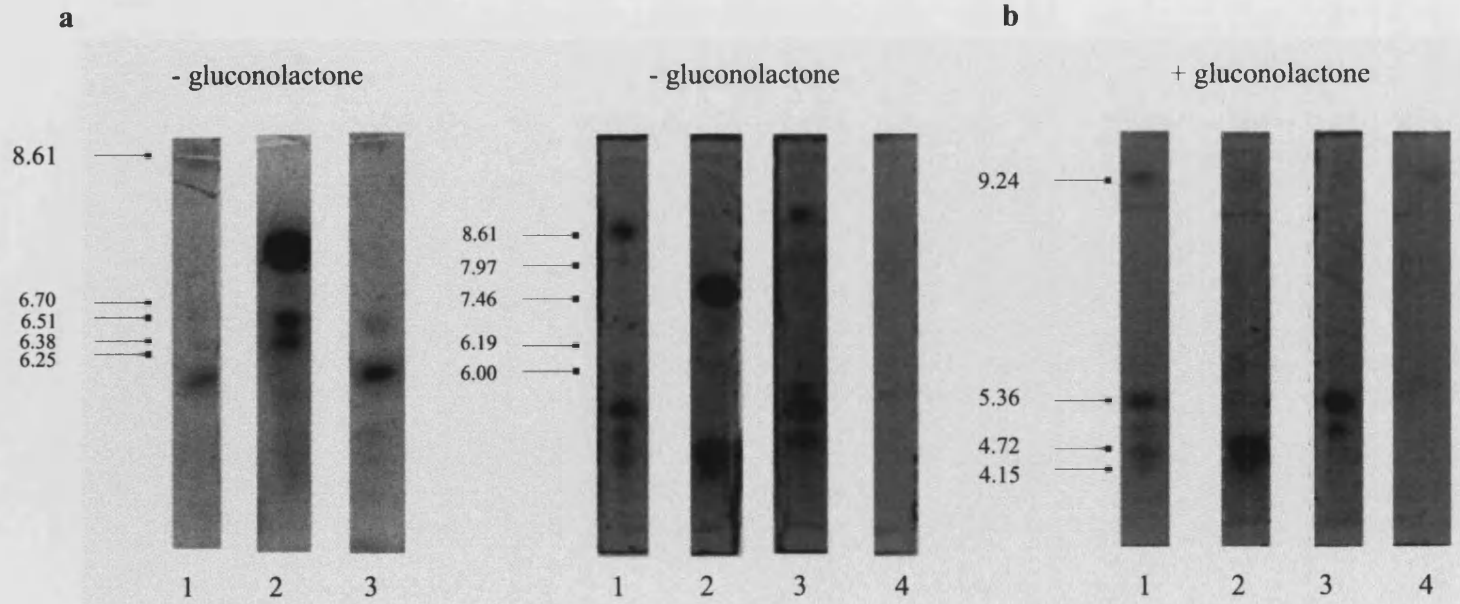


Figure 5.12: Isoforms of β-glucosidase and cellobiohydrolase secreted by *T. harzianum* genotypes in the presence of wheat straw. (a) 12 hours induction; (b) 24 hours induction. Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4).

*gluconolactone inhibited β-glucosidase activity.

5.1.3(iv) Isoform profiles of xylanases produced by T. harzianum genotypes in the presence of wheat straw

Endo-β-1,4-xylanase activity was detected using Remazol Brilliant Blue covalently linked to the substrate, 4-*O*-methyl-D-glucurono-xylan (RBB-xylan) (Sigma, UK). The procedure for activity gel overlay is described in Materials and Methods (see section 2.3.6).

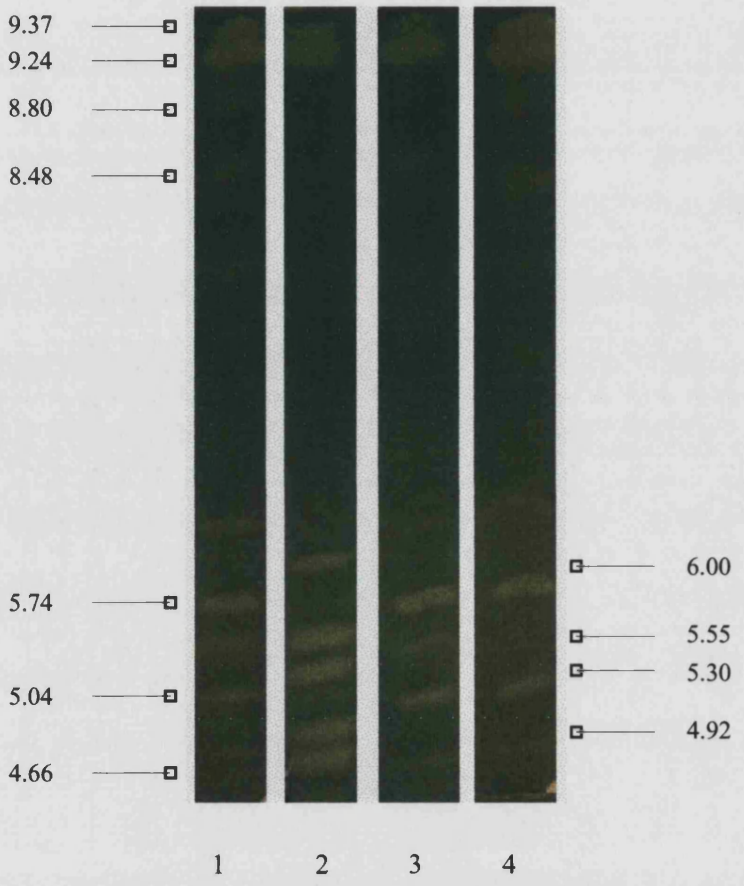
Induction for 12 hours led to the production of one xylanase isoform, common to all genotypes, with pI 9.24. After 24 hours induction additional isoforms were apparent (Fig 5.13). All four genotypes continued to secrete the isoform (pI 9.24) observed after just 12 hours. In addition, all genotypes also produced isoforms with pI's 4.66 and 9.37. Genotypes 1, 2 and 4 all produced isoforms with pIs 5.04, 5.74 and 8.80. Genotype 3 was unique in the secretion of isoforms with pI 4.92, 5.30, 5.55 and 6.00. Genotypes 1 and 2 also produced an isoform (pI 8.48) that appeared less active than others produced by same genotypes.

5.1.3(v) Isoform profiles of protease and endo-β-1,3-glucanases produced by T. harzianum genotypes in the presence of wheat straw

Wheat straw also contains mixed-linkage β-D-glucans that are not cellulosic (McNeil *et al.* 1984). These include β-1,3-glucans and could act as a matrix to stabilize the structural cellulose microfibrils. Therefore the culture fluids of *T. harzianum* exposed to wheat straw were also tested for endo-β-1,3-glucanase activity. Protease was detected using Remazol Brilliant Blue-gelatin (Loewe Biochimica GmbH), while Remazol Brilliant Blue-curdlan (Loewe Biochimica GmbH) was used to reveal endo-β-1,3-glucanase activity. The gels were produced as described in sections 4.1.4(i) and 4.1.4(iii). Activity was observed as achromatic bands on a blue background of intact substrate.

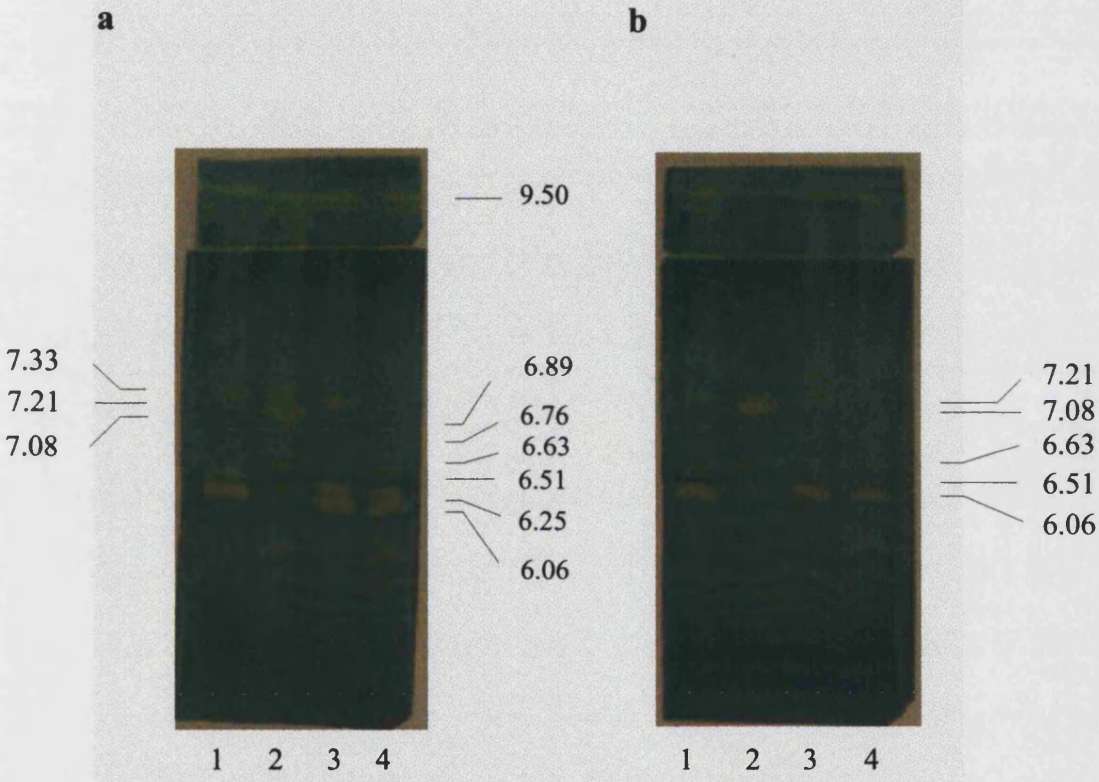
After 12 hours induction, all four genotypes secreted a protease with pI 9.50 (Fig 5.14a). Genotypes 1 and 3 (non-aggressive) produced very similar isoform profiles; both genotypes produced apparent protease isoforms with pIs of 6.06, 6.25 and 7.21. Genotypes 2 and 4 secreted isoforms identical to those of genotypes 1 and 3 (pI 6.06, 6.25 and 7.21), however these genotypes differed from the non-aggressive genotypes in the apparent amount of activity as considered by prominence of the clearing zone. The

Figure 5.13: Isoforms of xylanase by *T. harzianum* genotypes in the presence of wheat straw.



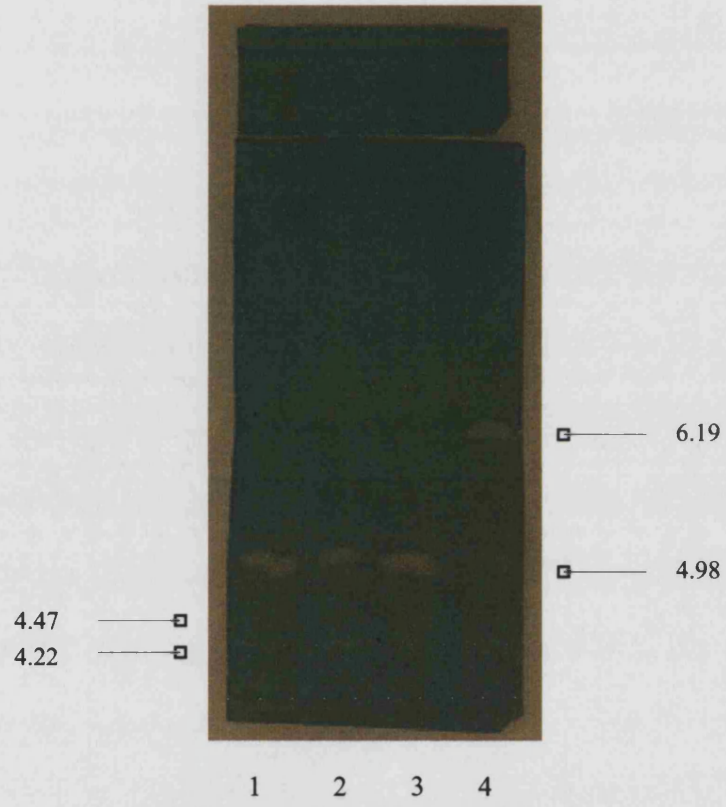
After 24 hours induction. Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4). Values denote pI as determined from a calibration curve.

Figure 5.14: Isoforms of protease secreted by *T. harzianum* genotypes in the presence of wheat straw.



(a) 12 hours induction; (b) 24 hours induction. Lanes: 1, Th1(c) (Th1); 2, T7 (Th2); 3, TD7 (Th3); 4, RM10c (Th4).

Figure 5.15: Isoforms of laminarinase secreted by *T. harzianum* genotypes in the presence of wheat straw.



After 24 hours induction. Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4). Values denote pI as determined from a calibration curve.

pI 7.21 isoform, appeared to be the dominant proteinase secreted by non-aggressive genotypes (1 and 3) and was visible as a faint achromatic band in the profiles of genotypes 2 and 4. While isoforms with pI 6.06 and 6.25 appeared to be predominantly produced by genotypes 1, 3 and 4, only a faint clearing zone was visible for genotype 2. In addition, genotype 2 also appeared to secrete several other proteases indicated by feint clearing zones, with pIs of 6.51, 6.63, 6.76, 6.89, 7.08 and 7.33.

Induction for 24 hours revealed fewer protease isoforms (Fig 5.14b). The principal isoforms of protease secreted were pI 6.06 by genotypes 1, 3 and 4, while genotype 2 predominantly produced an isoform with pI 7.08. All four genotypes continued to produce protease with pI 7.21, although the clearing zone was feint. Genotype 2 also continued to secrete pI 6.51 and 6.63 protease isoforms, with low level activity when compared to other clearing zones.

Laminarinase (endo-β-1,3-glucanase) activity was detected for all genotypes and the profile was very similar to the profiles detected in the presence of *A. bisporus* cell walls (Fig 5.15). Genotypes 1, 2 and 3 produced an apparent endo-β-1,3-glucanase isoform with pI 4.98. Genotype 4 (RM10c) also produced an isoform with pI 4.98 but the faint band indicated low activity. Genotype 4 was unique in the secretion of an endo-β-1,3-glucanase, pI 6.19. Isoforms with pIs of 4.22 and 4.47 were detected, though achromatic bands were very faint, for genotypes 1, 2 and 3.

5.1.4 Isoform profiles of depolymerases produced by T. harzianum genotypes on sterile mushroom compost

Trichoderma harzianum produced various depolymerases that could be instrumental in the degradation of wheat straw which served as sole source of carbon in *in vitro* cultures. The inoculation of sterile compost with *T. harzianum* spores and the retrieval of depolymerases from compost was necessary to prove that depolymerases could possibly be secreted in mushroom compost and therefore could suggest a saprophytic stage involved in the antagonism between *T. harzianum* and *A. bisporus*. This investigation provided a means of further testing the possible saprophytic abilities of *T. harzianum* genotypes.

Sterile compost (autoclaved at 121°C for 30 minutes, repeated twice – to eliminate heat activated organisms) was inoculated with rye grains rolled in *T. harzianum* spores. Ten grains were added to each tube, which was filled with *ca.* 30 g of sterile compost and the lid tightened and then unscrewed a quarter turn to provide aeration. Tubes were incubated at 25°C, in the absence of light. Three tubes were removed after successive incubation periods of 1, 2 and 3 weeks. Samples of compost were removed from each tube with a combined sample fresh weight of 50 g and care was taken to avoid compost surrounding the inoculum grains. Enzymes were retrieved in an extraction buffer (that should release and preserve enzymes) which consisted of 50 mM sodium phosphate buffer [pH 6.0] containing 5 mM dithiothreitol (prevented oxidation), 0.2 M NaCl (allowed desorption of proteins from cell walls) and 5% polyvinyl pyrrolidone (absorbed phenols) (Cooper and Wood 1980). Compost samples were extracted in 250 ml cold extraction buffer in a stomacher bag. Samples were agitated for 2 minutes in a Stomacher Lab-Blender 400 and then steeped on ice for 15 minutes. Subsequently samples were stomached for a further 2 minutes before filtration through two layers of muslin. Filtrates were clarified by centrifugation at 4 000 xg for 15 minutes and the resulting supernatants further clarified at 23 000 xg for 15 minutes. The supernatants were dialysed exhaustively against 25 mM MES [pH 6.0] at 4°C. Finally, extracts were concentrated *ca.* 5-fold in 30 000 M_r polyethylene glycol (PEG) at 4°C. Total protein content was measured using the BioRad assay (see Methodology section 2.2.9). Samples containing 100 μg protein were flash frozen in liquid nitrogen, lyophilised and stored at -20°C.

Extracellular enzyme activity after 2 weeks of incubation was assessed directly from compost extracts using Nelson-Somogyi assay for reducing sugars (see Materials and Methods 2.4.1). Total xylanase, cellulase, laminarinase and chitinase activities were determined (Fig 5.16 and Fig 5.17). Non-aggressive genotypes (1 and 3) exhibited higher activities of xylanase, cellulase and laminarinase than the aggressive genotypes (2 and 4). Only genotype 3 demonstrated substantial chitinase activity. Some activities were detected in extracts from the compost controls.

Protein samples were separated according to iso-electric points by gel (electrofocussing). Prior to electrofocussing the samples were passed through PD-10 columns (Pharmacia) to eliminate any salts not removed by dialysis (see Methodology section 2.2.2).

Enzyme activity of separated proteins was determined by activity overlays containing the relevant substrates. Cellulase and xylanase were detected by overlays containing Ostazin brilliant red-hydroxyethylcellulose (OBR-HEC) and Remazol brilliant blue-4-*O*-methyl-D-glucurono-xylan (RBB-xylan), respectively [see sections 5.1.3(iii) and (iv)]. Remazol brilliant blue-curdlan was incorporated to detect laminarinase activity [see section 4.1.4(i)].

Cellulase activity was detected by the appearance of achromatic zones on a red background of undegraded OBR-HEC and was demonstrated by all four genotypes (5.18a). Genotype 3 (TD7) produced abundant cellulase isoforms with a broad range of iso-electric points, *ca.* 15 isoforms with pI range 4.43 to 8.99. All genotypes produced an isoform with pI 4.69. Genotype 1 (Th1(c)) appeared to secrete two additional isoforms with pIs 6.97 and 7.30.

Non-aggressive genotypes (1 and 3) appeared to produce substantially more xylanase isoforms than aggressive genotypes (Fig 5.18b). Genotype 3 produced the most xylanase isoforms, which spanned a broad pI range: 4.76 to 9.38. Isoforms common to all genotypes included those with pIs 6.25 and 6.91. Genotypes 1, 3 and 4 produced an isoform with pI 7.36. In addition, the non-aggressive genotypes produced two highly alkaline isoforms, pI 9.38 (Th3) and 9.51 (Th1). Genotypes 1 and 4 produced an isoform with pI 8.92. An isoform with pI 4.95 was secreted by genotypes 2 and 3. Genotype 2 was apparently unique in the secretion of a pI 9.06 xylanase isoform.

The achromatic zones depicting laminarinase activity were less discrete than those for cellulase and xylanase activity. Genotype 3 appeared to produce the most isoforms of laminarinase (Fig 5.18c). The majority of isoforms secreted by aggressive genotypes (2 and 4) had acidic pIs. Genotype 4 secreted an isoform with pI 6.25; possibly identical to the isoform produced in *in vitro* culture in the presence of *A. bisporus* cell walls or wheat straw. In addition, genotypes 2 and 4 demonstrated activity of two isoforms with pI's 5.67 and 5.99. All genotypes produced laminarinase isoforms with pI 4.95 and 5.15. Non-aggressive genotypes were unique in the secretion of alkaline laminarinase isoforms in the pI range 7.23 to 8.08 and 6.51 to 8.21 for genotypes 1 and 3 respectively.

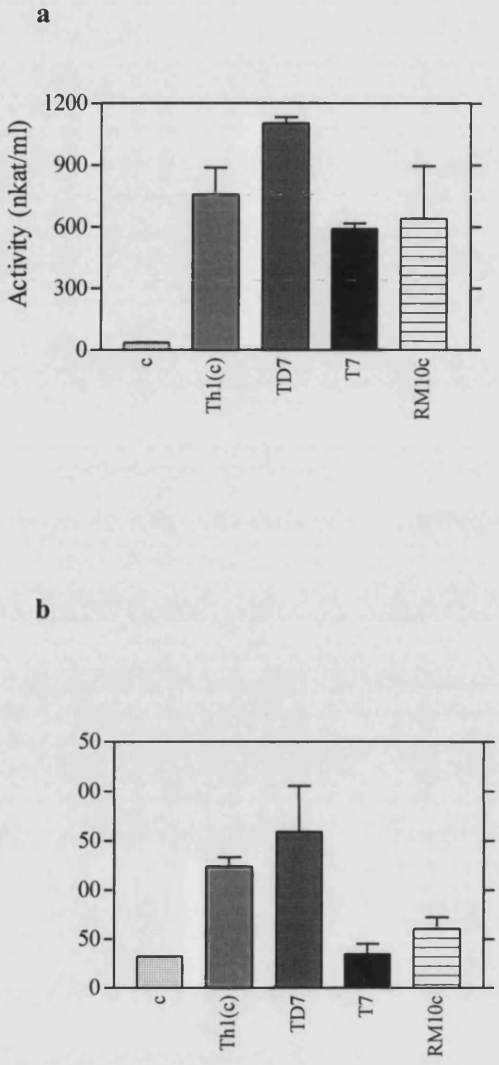


Figure 5.16: Extracellular enzyme activity produced by *T. harzianum* isolates in sterile mushroom compost after 2 weeks of incubation: a) xylanase activity and b) cellulase activity. Bars indicate standard error of the mean of three replicates.

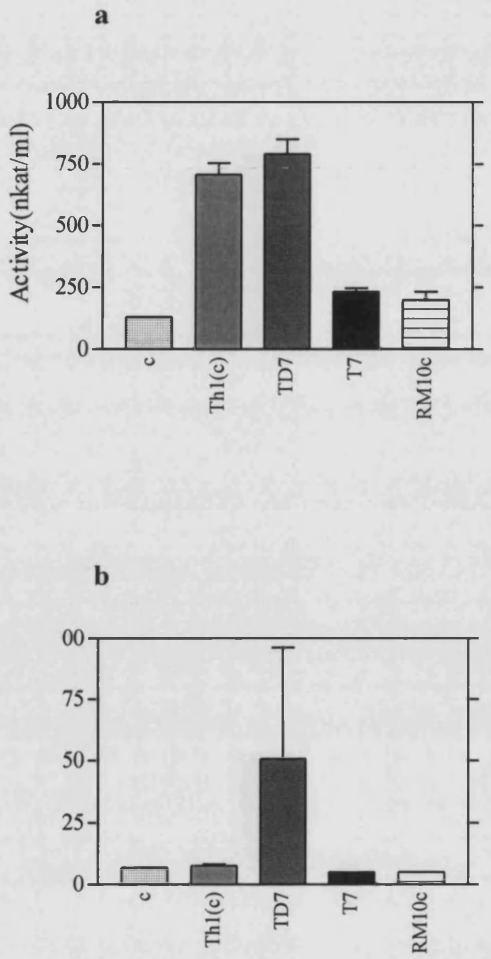
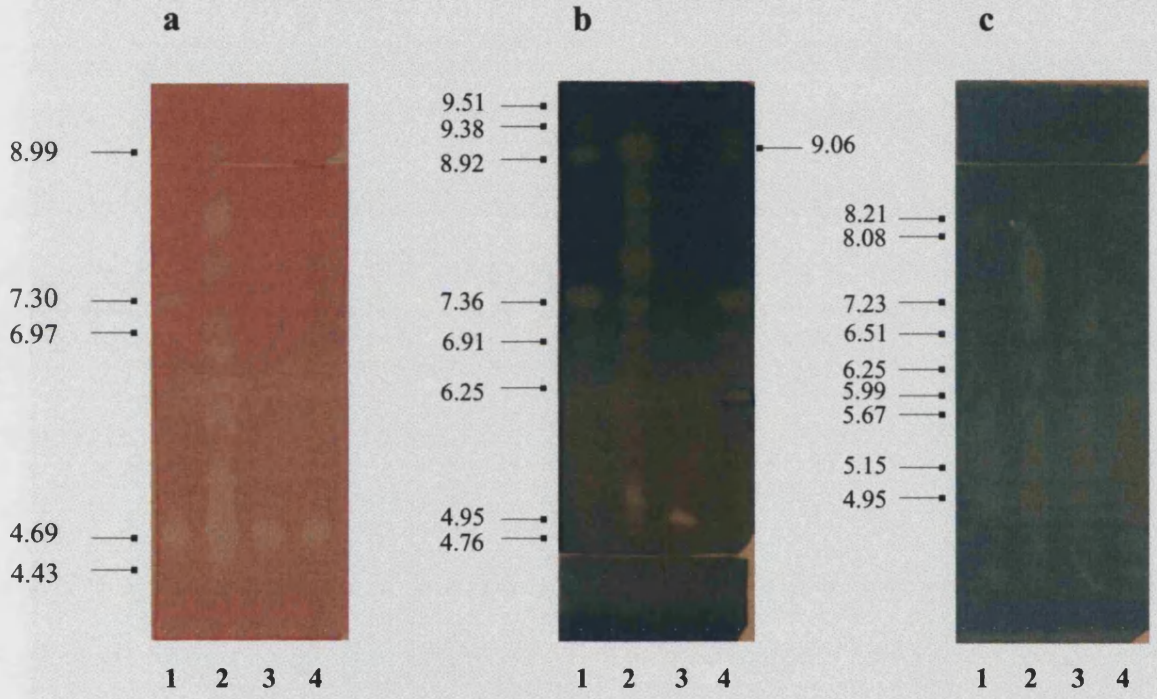


Figure 5.17: Extracellular enzyme activity produced by *T. harzianum* isolates in sterile mushroom compost after 2 weeks of incubation: a) laminarinase activity and b) chitinase activity. Bars indicate standard error of the mean of three replicates.

Figure 5.18: Isoforms of depolymerases secreted by *T. harzianum* genotypes in sterile mushroom compost.



Activity visible as achromatic zones on a coloured background. (a) Cellulase activity. (b) xylanase activity. (c) Laminarinase activity. Lanes: 1, Th1(c) (Th1); 2, TD7 (Th3); 3, T7 (Th2); 4, RM10c (Th4).

5.1.5 Preliminary development of a potential diagnostic test for aggressive antagonism in strains of T. harzianum

Diagnosis of aggressive compost-colonising strains of *T. harzianum* may be classified by molecular analysis, however these strains are often identified by the more traditional methods of growth characteristics and tube colonisation assays. The molecular techniques used to date distinguish genotypes by comparison of restriction fragment length polymorphism analysis (RFLP) and random amplified polymorphic DNA (RAPD) using ribosomal DNA (Muthumeenakshi *et al.* 1994, 1998; Ospina-Giraldo *et al.* 1997a, 1997b; Chen *et al.* 1997). Both the growth characteristics and molecular techniques are time consuming and require substantial experience with many strains of *T. harzianum*. Therefore the development of a simple and rapid test that distinguishes aggressive *T. harzianum* strains from other genotypes and other species would allow more effective containment and control of green mould outbreaks. Furthermore, if the test could be developed for the polymerase chain reaction (PCR), results could be obtained rapidly from a small amount of sample material. A preliminary PCR diagnostic test, based on primers designed to a cellulose-growth-specific protein gene (*cell*) of *A. bisporus* (Raguz *et al.* 1992) was developed at HRI Wellesbourne (per. comm. Peter Mills, HRI Wellesbourne).

5.1.5(i) Optimisation of the PCR method for use with cell primers and T. harzianum genomic DNA

Primers previously designed to *cell* gene of *A. bisporus* were used initially, however the forward primer spanned an intron and therefore amplification was hindered. A new forward primer was designed using PrimerCalc™ (Molecular Sensing Plc, UK). The new primer pair amplified a product of *ca.* 500 bp from *A. bisporus* genomic DNA. Optimisation was achieved using Taguchi analysis which allowed the investigation of four variables in nine reactions (Cobb and Clarkson 1994).

The First Step™ kit (Molecular Sensing Plc, UK) was used, consisting of nine pre-prepared reaction solutions containing: reaction buffer, $MgCl_2$, dNTPs and Taq polymerase in a volume of 30 µl. Primers, template (1.0 µl, 10 ng μl^{-1} DNA) and milliQ water were added in a volume of 20 µl. The variable components were primers (10, 20 and 30 pmoles), dNTPs (100, 200 and 300 µM), $MgCl_2$ (1.0, 2.0 and 3.0 mM) and Taq polymerase (0.5, 1.25 and 2.0 units). The reactions were run on a thermal program designed by FirstStep™: denaturation at 94°C for 30 seconds, 2 minutes at an annealing temperature of 50°C

followed by extension for 30 seconds at 72°C. This sequence was repeated for 35 cycles. Products for each reaction were scored between 1 and 10 and subsequently optimal concentrations for components were calculated (see Materials and Methods 2.5.2).

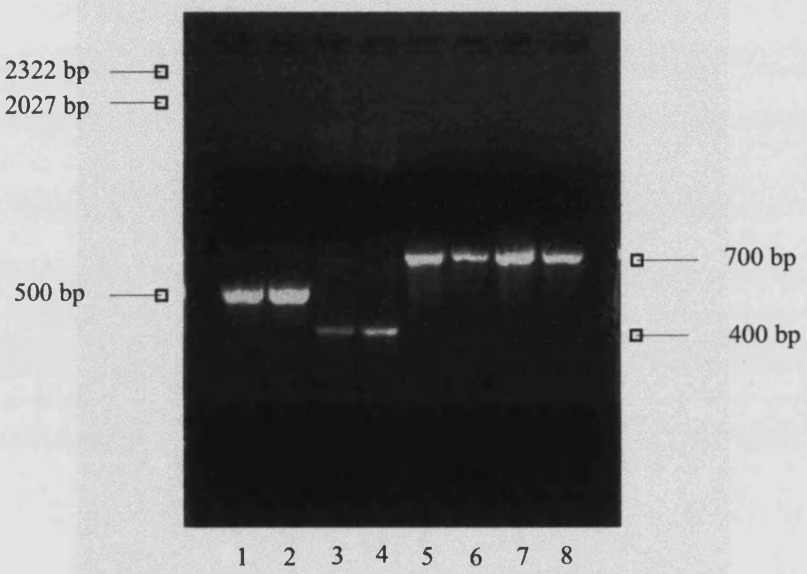
PCR components were optimised in the same manner for *T. harzianum* genomic DNA. Optimal component concentrations were 100 μM dNTP, 1.0 mM $MgCl_2$, 20 pmole primers and 2.0 units Taq. *A. bisporus* and *T. harzianum* genomic DNA was amplified by the same PCR program described above.

Amplification of *A. bisporus* genomic DNA resulted in a 500 bp product, while aggressive genotypes of *T. harzianum* (Th2 and Th4) produced a larger amplification fragment (*ca.* 700 bp). Genotype 1 exhibited an amplification product smaller than that of *A. bisporus* (*ca.* 400 bp); the product also demonstrated lower intensity staining suggesting a low yield (Fig 5.19). Genotype 3 produced two, low yielding amplification fragments; one product was very similar in size to the single product of the aggressive genotypes and the other product was larger (Fig 5.20). This test appeared to distinguish between non-aggressive and aggressive genotypes of *T. harzianum*, as well as *A. bisporus*.

The test was expanded to include more strains of the aggressive genotypes. While strains of genotype 4 were consistent in the production of a 700 bp product, genotype 2 exhibited some variation. Three strains of genotype 2 (KPNT, Th2F and T32) displayed an amplification product of similar size to genotype 1. In addition, strains KPNT and Th2F also exhibited the 700 bp product associated with other aggressive strains, although the former amplified the fragment in substantially lower yield than the latter (Fig 5.20). Genotype 3 strains produced two amplicons *ca.* 700 and 850 bp.

The selectivity of PCR amplification with the *cel1* primers was investigated with genomic DNA from other organisms. Conditions for *T. harzianum* reactions were used with genomic DNA from the fungi *Metarhizium anisopliae* (ME1), *Stagonospora nodorum*, *Saccharomyces cerevisiae* and *Spongospora subterranea* f. sp. *nasturtii*/Watercress (kindly donated by members of the Microbial Pathogenicity Group, University of Bath). A reaction containing *A. bisporus* DNA was included as a positive control. After gel analysis only *A. bisporus* exhibited a discrete product (Fig 5.21). *S. nodorum* exhibited a smear and all other organisms failed to demonstrate any amplification product.

Figure 5.19: Diagnostic PCR products from genomic DNA from *T. harzianum* genotypes and *A. bisporus* genomic DNA



Lanes: 1-2, *A. bisporus*; 3-4, Th1(c) (Th1); 5-6, T7 (Th2); 7-8, RM10c (Th4).

Figure 5.20: Diagnostic PCR products from *T. harzianum* genotypes.

Lanes: 1and 18: HindIII Lambda DNA; 2, *A. bisporus*; 3, Th1(c) (Th1); 4, TD15 (Th1); 5, T28JF (Th1); 6, TD7 (Th3); 7, Th3c (Th3); 8, A006022 (Th3); 9, T7 (Th2); 10, Th2A (Th2); 11, KPNT (Th2); 12, T7red (Th2); 13, Th2F (Th2); 14, T32 (Th2); 15, RM10c (Th4); 16, BE (Th4); 17, RM10m (Th4).

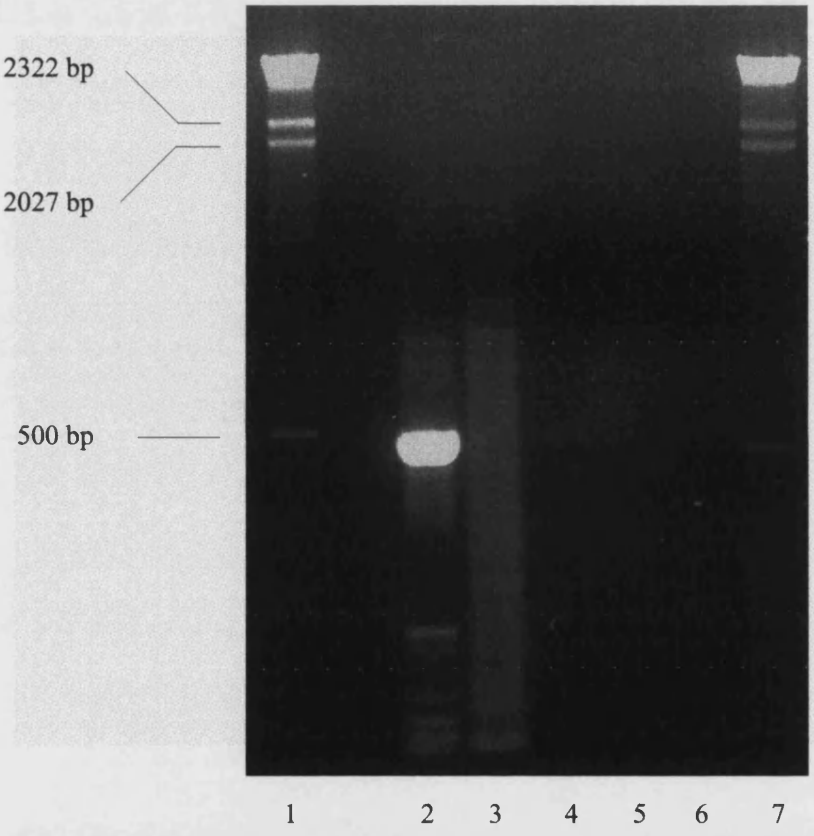
5.1.5(ii) Sequencing of PCR products and comparison of T. harzianum genotype sequences

Amplicons produced from genomic DNA of strains of *T. harzianum* with primers designed to *cel1* gene of *A. bisporus* could distinguish strains according to amplicon size. However, variability within genotype 2 presented the possibility of false negative results. Genotype 2 strains that produced both the small product and the 700 bp product could be distinguished from genotype 1. Strain T32 (Th2) failed to produce the 700 bp product and only produced an amplicon of similar size to non-aggressive genotype 1.

In a preliminary study the products of *A. bisporus* and *T. harzianum* genotypes 1 (Th1(c)), 2 (T7) and 4 (RM10c) were sequenced to allow product comparisons. Products were purified from the agarose gel using a Sephaglas™ BandPrep Kit (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. A sample (5 µl) of purified PCR product was quantified by comparison with known standards of uncut lambda phage DNA on a 1.5% w/v agarose gel in 0.5x TBE. Sequencing reactions contained 30 ng PCR product DNA and 3.2 pmole forward primer in a total volume of 6 µl.

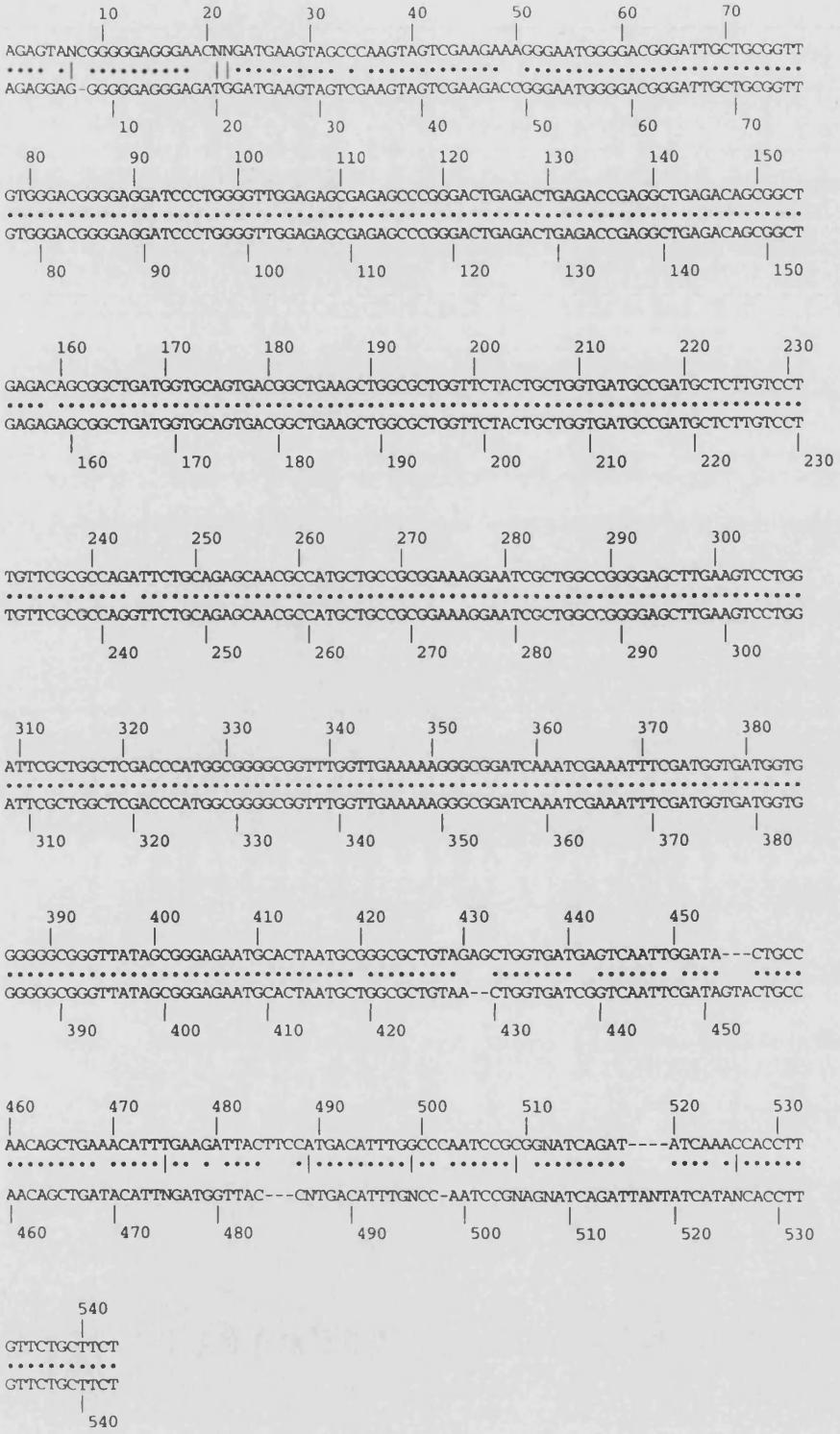
The amplification product sequence from *A. bisporus* genomic DNA was submitted as a query to the Blast database. The database detected 98% identity between the amplification product and a fragment of *A. bisporus* cellulose-growth-specific protein gene (*cel1*). The sequences from the PCR products of *T. harzianum* exhibited no significant similarity to the *A. bisporus* amplicon. The *T. harzianum* sequences showed no similarity to other sequences on the database. Comparison of sequences from genotypes 2 and 4 (aggressive) revealed 92% identity (Fig 5.22) in contrast genotype 1 (Th1(c)) exhibited no significant identity with aggressive *T. harzianum* genotypes.

Figure 5.21: Evaluation of specificity of the PCR diagnostic test by addition of genomic DNA extracted from other organisms.



Lanes: 1 and 7, HindIII Lambda DNA; 2, *A. bisporus*; 3, *Spongospora* f. sp. *nasturtii*/ watercress; 4, *M. anisopliae* (ME1); 5, *Stagonospora nodorum*; 6, *Saccharomyces cerevisiae*.

Figure 5.22: Amplicon sequence homology of aggressive genotypes of *T. harzianum* associated with mushroom compost.



T. harzianum isolates: top, T7 (Th2); bottom, RM10c (Th4).

5.2 Discussion

Experiments in this work designed to investigate the potential for mycoparasitic behaviour of *T. harzianum* also revealed the ability for saprophytic growth in compost. Therefore studies to quantify the saprophytic growth of *T. harzianum* and to compare saprophytic abilities were designed. Reduction in mushroom yields caused by aggressive compost-colonising genotypes of *T. harzianum* may be directly related to saprophytic growth capabilities.

5.2.1 Saprophytic growth capabilities of T. harzianum genotypes

The TSM adapted from Askew and Laing (1993) reduced the growth rates of all strains to the extent that tight colonies formed which were easily assessed (Fig 5.1). This medium was highly selective for *T. harzianum* and no contaminants were observed from the compost controls.

Development of the selective medium was critical as mushroom compost contains an extensive microflora. Several media have been described that are selective for *T. harzianum* (Elad *et al.* 1981; Papavizas and Lumsden 1982; Elad and Chet 1983; Askew and Laing 1993). *Trichoderma* selective medium (TSM) was chosen to work with, as the fungicides were still available and in the formulations reported by Askew and Laing (1993). The addition of antibiotics (chloramphenicol and streptomycin) and the growth inhibitor, sodium deoxycholate (medium 1) reduced the growth rate of both strains. Nystatin substantially reduced growth and Th1(c) growth was not detected until after 12 days incubation; whereas isolate T7 growth was detected after 6 days incubation. The reasons for the growth differences of *T. harzianum* strains in the presence of these components are not clear.

MEA with fungicides and antibiotics substantially reduced the growth rates of both *T. harzianum* strains, typically 1.89-fold and 1.59-fold for strain Th1(c) and T7 respectively. Th2 had a higher tolerance of these fungicides. Askew and Laing (1993) included captan to eliminate *Fusarium* spp., which are not usually present in unspawned compost as was used here (Fermor TR, HRI Wellesbourne, per. comm.). The revised TSM used here excluded captan, which inhibited germination of *T. harzianum* conidia. Germination was critical, since many of the cfu retrieved were as spores. *Trichoderma* spp. are tolerant of high levels

of quintozene and rose bengal (Elad *et al.* 1981) and aggressive genotypes appeared more tolerant than did non-aggressive strains. Growth characteristic assessment on the selective medium of both aggressive and non-aggressive strains was essential so that there was no inherent variability due to a single component of the medium and to ensure cfu counts were an accurate representation of the saprophytic growth.

Three separate experiments confirmed the enhanced saprophytic abilities of Th2 (*ca.* 6.81-fold), Th3 (*ca.* 8.71-fold) and Th4 (*ca.* 7.52-fold) compared with Th1, which displayed significantly lower growth. To the author's knowledge this is the first report of saprophytic growth by *T. harzianum* genotypes associated with mushroom compost and could be the basis of genotype differences. Th3 differed from Th1 throughout the entire investigation even though both genotypes were previously classed as non-aggressive colonisers of compost with no reductions in *A. bisporus* yields (Seaby 1987; Muthumeenakshi *et al.* 1994). Th3, while indigenous to Europe and non-aggressive, exhibited greater divergence from Th1 than did Th2 (Muthumeenakshi *et al.* 1994). The enhanced saprophytic growth of Th2, Th3 and Th4 may be linked to the possible production of depolymerases which are more efficient in the degradation of compost components than those of Th1. Tolerance of compost bacteria is unlikely as previous investigations of the growth of *T. harzianum* strains on agar plates seeded with various bacteria isolated from compost failed to reveal tolerance of any *T. harzianum* strains to compost bacteria (Seaby 1996b).

Growth as cfu determined from the grain inoculum source was higher than that retrieved after saprophytic growth, probably because cfu were readily released from grain by 100 mM sodium tetrapyrophosphate. Therefore the growth after incubation in compost was determined from areas which did not contain the inoculum source and as such was indicative of mycelial growth and sporulation into a different area of compost. The retrieval of cfu from the mycelial network, which had permeated the straw component of the compost, was difficult but the mechanical procedure chosen allowed the fragmentation of the mycelium.

Once a phenotypic difference between saprophytic growth abilities of genotypes had been determined, the study focussed on possible explanations for this difference.

5.2.2 Depolymerases produced by T. harzianum genotypes on exposure to wheat straw

Depolymerases that could be involved in saprophytic growth were detected in the presence of wheat straw in *in vitro* culture fluids. Determination of total activity of cellulase and xylanase failed to reveal any differences between aggressive and non-aggressive genotypes. All genotypes displayed a rapid increase in rate of activity after an initial lag phase during the first 12 hours induction. However, in mushroom compost the efficacy and isozymes of depolymerases may differ according to *T. harzianum* genotypes.

Separation of depolymerase isoforms in free solution using IEF (Rotofor™) revealed that the majority of cellulase and xylanase activities had the same pI range for each depolymerase for all strains tested. Th2 and Th4 (aggressive) displayed xylanase isoforms with substantially higher activities than those of non-aggressive strains, typically 4.17-fold and 1.43-fold higher than Th1 and Th3, respectively. This is particularly relevant to saprophytic growth as hemicellulose is present in compost as *ca.* 23% of the dry weight of wheat straw (Fermor TR, HRI Wellesbourne, pers. com.) and *ca.* 75% of cereal grain cell wall (Kent 1983). Hemicellulose is less recalcitrant than cellulose and lignin, therefore high xylanase activities could be less critical for efficient degradation and thereby saprophytic growth of Th2 and Th4. Notably, Th3 exhibited higher cellulase and xylanase activities than Th1 (2.67-fold and 2.92-fold, respectively). This difference was also visible from activity overlays on IEF gels, since Th3 produced more isoforms than the other genotypes that may, in part, contribute to the enhanced saprophytic growth.

Activity overlays on IEF gels revealed the production of several endo-β-1,4-glucanases and β-glucosidases by Th1, Th2 and Th3 (but not Th4) after 12 hours induction. The failure to produce cellobiohydrolases at this time could suggest that the other cellulase activities were constitutive because EGII and CBHII of *T. reesei* are thought to be important for the production of soluble inducer; the slow release of cellobiose from cellulose triggers further cellulase gene expression (Seiboth *et al.* 1997; Kubicek 1993). This lack of cellobiohydrolase isoforms could therefore explain the low activity of endoglucanase and β-glucosidase. β-glucosidase activity may initially prevent the induction of the cellulase system due to degradation of the potential inducer, since cellobiose released in small quantities and absorbed intact, acts to induce the cellulase

system in *T. reesei* (Kubicek 1993). Also the cleavage of cellobiose into glucose could repress the expression of the cellulase system.

The alkaline β-glucosidase isoform (pI 8.61) secreted by Th1 and Th2, was detected after 12 hours. In contrast, Todorovic *et al.* (1990) reported that *T. harzianum* (C_1R_1) also in the presence of wheat straw, produced β-glucosidase isoforms in the pI range 4.85 to 7.50. Non-aggressive genotypes in this work did produce other β-glucosidase isoforms in this pI range (see Table 5.6), which may be similar to those previously detected.

An endo-β-1,4-glucanase isoform with a pI of 7.97 was specific to aggressive genotypes (2 and 4). This isoform may be similar to a low M_r endoglucanase characterised from *T. reesei* with a pI of 7.5 (Hayn *et al.* 1993) or EGIII, pI 7.7, of *T. reesei* (reviewed Goyal *et al.* 1991). This may be significant since, in contrast, several endo-β-1,4-glucanases produced by *T. reesei* (reviewed Goyal *et al.* 1991) and *T. harzianum* in the presence of wheat straw had acidic pIs (see Table 5.6). IEF activity overlays revealed unique cellulase and xylanase profiles for Th3 after 24 hours induction. More cellulase and xylanase isoforms were detected for Th3 when compared to the other genotypes making Th3 distinctive.

Genotypes 2, 3 and 4, which all exhibited higher saprophytic growth than did Th1, produced a range of endo-β-1,4-glucanases (pI 5.55 to 6.32) not secreted by Th1. The pI range of these additional endoglucanases is very similar to EGII (pI 5.5) from the cellulase system of *T. reesei* (reviewed Goyal *et al.* 1991). Under different conditions *T. harzianum* produced endo-β-1,4-glucanase isoforms of pI 6.4 and 7.6 in response to the presence of *Pythium ultimum* (Thrane *et al.* 1997). Additionally, *T. harzianum* secreted an endo-β-1,3-glucanase isoform (pI 5.0) in the presence of *P. ultimum* (Thrane *et al.* 1997) and here *T. harzianum* genotypes produced isoforms of endo-β-1,3-glucanase with similar pIs, in the presence of *A. bisporus* cell walls and wheat straw. Perhaps mixed-linkage glucans from cereal cell walls (McNeil *et al.*1984; Carpita and Gibeaut 1993) could stimulate similar isoforms under the different circumstances of mycoparasitism and saprotrophism.

Table 5.6: Summary of genotype-distinguishing depolymerase isoforms produced *in vitro*.

Depolymerase	*T. harzianum* genotype	Isoelectric point	Previously reported
Endo-β-1,4-glucanase	Th1	5.49, 6.44, 7.78, 8.03	7.5 (Hayn *et al.* 1993)
	Th3	3.96 to 7.02 (6.06, 6.19, 6.32)	5.5 (EGII *T. reesei* reviewed Goyal *et al.* 1991) 6.4, 7.6 (Thrane *et al.* 1997)
	Th2	5.55, 5.74, 7.97	as Th1 and Th3
	Th4	5.68, 5.87, 7.97	as Th1 and Th3
CBH	Th1	4.85, 5.36, 9.24	4.2, 5.9 (CBHI and II *T. reesei* Gama *et al.* 1998)
	Th3	4.15, 4.72	as Th1
	Th2	5.36, 9.24	as Th1
	Th4	5.36, 9.24	as Th1
β-glucosidase	Th1	6.00, 7.97, 8.61	4.85 to 7.50 (Todorovic *et al.* 1990)
	Th3	6.19, 7.46	as Th1
	Th2	8.61	
	Th4	8.29	
Endo-β-1,4-xylanase	Th1	9.37, 9.24, 4.66 5.04, 5.74, 8.80, 8.48	9.0, 5.2 to 5.5 (Torronen *et al.* 1992; Tenkanen *et al.* 1992)
	Th3	9.37, 9.24, 4.66 4.92, 5.30, 5.55, 6.00	as Th1
	Th2	9.37, 9.24, 4.66 5.04, 5.74, 8.80, 8.48	as Th1
	Th4	9.37, 9.24, 4.66 5.04, 5.74, 8.80	as Th1
Laminarinase	Th1	4.22, 4.47, 4.98	5.0 (Thrane *et al.* 1997)
	Th3	4.22, 4.47, 4.98	as Th1
	Th2	4.22, 4.47, 4.98	as Th1
	Th4	4.98, 6.19	as Th1 6.0 (Vazquez-Garciduenas *et al.* 1998)
Protease	Th1	9.50, 6.06, 6.25, 7.21	10.3 (Prl of *M. anisopliae* St Leger *et al.* 1987) 9.2 (Prb1 Geremia *et al.* 1993)
	Th3	9.50, 6.06, 6.25, 7.21	as Th1
	Th2	9.50, 6.06, 6.25, 7.21 6.51,6.63, 6.76, 6.89, 7.08, 7.33	as Th1
	Th4	9.50, 6.06, 6.25, 7.21	as Th1

Not all isoforms produced are quoted; only those common to several genotypes or to aggressive or non-aggressive are given. Previously reported isoforms were produced by *T. harzianum* unless otherwise stated.

Th4 displayed β-glucosidase activity after 24 hours induction, indicating that these enzymes may only be triggered after the induction of endoglucanase and cellobiohydrolase to cleave the disaccharide products of crystalline cellulose. Alkaline β-glucosidase isoforms produced here by Th1, Th2 and Th4 were quite different to those reported from a Canadian strain of *T. harzianum* under similar culture conditions (Todorovic *et al.* 1990). Additional isoforms secreted by Th1 and Th3 after 24 hours induction were different to those produced after 12 hours, suggesting that earlier detected isoforms may be under the control of a different regulation mechanism. Alternatively the different isoforms may be artefactual and may derive from the earlier forms. Th1 and Th3 produced β-glucosidase isoforms (pI range 6.0 to 7.97) which have some similarity with β-glucosidase isoforms produced by *T. harzianum* C_1R_1 (Todorovic *et al.* 1990).

All genotypes produced two isoforms of cellobiohydrolase (Th1 secreted an additional isoform) which corresponds to the two CBHs of the *T. reesei* cellulase system (reviewed by Wood and Garcia-Campayo 1990; Goyal *et al.* 1991). Genotypes of *T. harzianum* associated with mushroom compost here, secreted CBH with pIs similar to those reported for *T. reesei* by Gama *et al.* (1998) ie. 4.2 and 5.9 for CBHI and CBHII respectively. In addition genotypes 1, 2 and 4 produced a highly alkaline isoform (pI 9.24) of CBH which has not be previously reported. Possibly the alkaline CBH isoform is specifically produced in *T. harzianum* genotypes associated with the alkaline pH of mushroom compost. All genotypes secreted more isoforms of endoglucanase than of CBH and this resembles the *T. reesei* cellulase system which contains six endoglucanases and two CBHs (reviewed Wood and Garcia-Campayo 1990). The degradation of cellulose to mono- and di-saccharides was enhanced by the synergistic action of EGI and CBHII (exo-acting) from *T. reesei* (Kleman-Leyer *et al.* 1996). The efficient degradation of wheat straw would require a full complement of cellulases and all genotypes of *T. harzianum* have displayed the ability to secrete the three classes of cellulase. The production of additional endoglucanases by genotypes 2, 3 and 4 could be responsible for the enhanced saprophytic growth of these genotypes in compost. Additional endoglucanases could enable more efficient degradation and provide more accessible bonds for CBH exo-activity.

Endo-β-1,4-xylanase isoforms displayed more uniformity than cellulases amongst the genotypes. The production of very similar isoform profiles by *T. harzianum* genotypes suggests that hemicellulolytic action may be vital for the degradation of wheat straw and

rye grain. Hemicellulose constitutes the matrix component of cereal cell walls and as such binds to and bridges the cellulose microfibrils conferring strength to the cell wall structure (McNeil *et al.* 1984; Carpita and Gibeaut 1993). Therefore efficient degradation of the matrix will lead to rapid deconstruction of the plant cell wall increasing accessibility of other polymers. However xylanase activity may not be essential for saprophytic growth in mushroom compost because Th1 did not differ from the other genotypes with regards to xylanase activity. The combination of xylanases with the additional endoglucanase isoforms specific to aggressive genotypes could result in synergy that may be responsible for enhanced saprophytic growth.

The first xylanase isoform detected here from all genotypes of *T. harzianum* in the presence of wheat straw had a pI of 9.24 and may be similar to XYNI (pI 9.0) of *T. reesei* (Torronen *et al.* 1992; Tenkanen *et al.* 1992). *T. harzianum* genotypes also produced several acidic isoforms in the presence of wheat straw that had pIs similar to XYNII (pI 5.2 to 5.5) from *T. reesei* (Torronen *et al.* 1992; Tenkanen *et al.* 1992). The saprophytic growth of Th1 was limited (mainly as numerous spores localised around the inoculum grain) and contrasted with that of the other genotypes. Lacking the additional endoglucanases detected from the other genotypes, Th1 may be unable to colonise the compost by 'exploratory' growth and therefore reverts to ruderal behaviour by directing resources from hemicellulase activity into an 'escape' mechanism common to ruderals i.e. the production of vast numbers of reproductive propagules (Pianka 1970).

A xylanase and a CBH isoform produced by genotypes 1, 2 and 4 shared the same pI of 9.24. This could be two separate depolymerase isoforms that have migrated to the same isoelectric point, or it could represent a complex (Biely *et al.* 1985b), or the isoform may have low specificity, capable of mixed degradative activities. EGs and CBHs of *T. reesei* displayed low xylanase activity (Gama *et al.* 1998).

Protease isoforms secreted by *T. harzianum* in the presence of wheat straw revealed some isoforms with isoelectric points similar to those produced in the presence of *A. bisporus* cell walls (see Chapter 4). However, overall the profiles induced by wheat straw revealed fewer isoforms than the *A. bisporus* cell wall-induced forms. *A. bisporus* cell walls triggered the secretion of additional isoforms but that appeared to be less active than those from the straw cultures (as judged by the prominence of the clearing zones). The

stimulation of more proteases by mushroom cell walls may indicate a more diverse protein profile associated with this cell wall extract. All genotypes secreted an isoform with pI 6.25 which could be similar to the pI 6.22 isoform secreted by aggressive genotypes in the presence of *A. bisporus* cell walls.

Th1 and Th3 produced identical protease isoforms after 12 and 24 hours induction with several predominant isoforms (pI 6.06, 6.25 and 7.21). Aggressive genotypes showed some similarity with proteases of the non-aggressive isolates, but the aggressive genotypes were quite different to each other. Of the three genotypes (2, 3 and 4) that exhibited enhanced saprophytic growth in compost, only Th3 and Th4 produced common dominant isoforms of protease (pI 6.06 and 6.25). This could suggest that proteases have a less important role in this form of growth or that all genotypes are efficient at obtaining and assimilating sources of nitrogen from compost. *A. bisporus* exhibited serine protease activity in mushroom compost which increased during mycelial growth (Burton *et al.* 1997). This protease appeared to be important in the degradation of protein in compost, the majority of which is bound to the lignin-humic complex. The involvement of laccases in the degradation of this complex is also extremely important and this is demonstrated by the rapid increase of laccase concentration during mycelial colonisation of compost by *A. bisporus* (Wood and Goodenough 1977). Although *T. harzianum* genotypes are capable of producing protease activity for the potential degradation of protein content of wheat straw, the failure to exhibit ligninase activity (see Appendix A.6) suggests that *T. harzianum* would be at a disadvantage in the degradation of the lignin-humic complex. Potentially, as a soil saprotroph, *T. harzianum* could be an 'opportunist' with efficient up-take systems and therefore could scavenge the degradation products of ligninases, laccases and proteinases of *A. bisporus*. Unaccompanied, the profiles of protease isoforms may not explain the differences in saprophytic growth by *T. harzianum* genotypes, however in combination with other genotype-specific depolymerase isoforms synergistic action may enable enhanced saprophytic growth.

The *T. harzianum* genotype laminarinase isoform profiles revealed differences according to geographical origin and were very similar to those produced in the presence of *A. bisporus* cell walls. The predominant isoform produced by Th4 was more alkaline (pI 6.19) than those produced by others genotypes (pI 4.98). Thus, while laminarinase activity was induced by wheat straw and by *A. bisporus* cell walls and is necessary for the efficient

degradation of mixed β-glucans, this activity does not appear to determine the degree of saprophytic ability. The laminarinase activity of *A. bisporus* is not associated with fruiting as are some depolymerases such as cellulase (Wood and Goodenough 1977). Also laminarinase activity may be significant in combination with other cell wall-degrading enzymes in saprophytic growth. Here the predominant isoform secreted by Th1, Th2 and Th3 (pI 4.98), may be similar to a laminarinase isoform (pI 5.0) of *T. harzianum* produced in response *Pythium ultimum* (Thrane *et al.* 1997). Vazquenz-Garciduenas *et al.* (1998) reported that *T. harzianum* secreted a laminarinase isoform (pI 6.0) under conditions designed to simulate mycoparasitism and this could be similar to a dominant isoform (pI 6.19) produced here by the North American genotype (Th4). Similarities with endoglucanases secreted during mycoparasitism could suggest that the different growth strategies require similar depolymerase isoforms, but which may be induced by different recognition events.

5.2.3 Depolymerases produced by T. harzianum genotypes on exposure to sterile compost

Depolymerases were retrieved from sterile compost inoculated with *T. harzianum* genotypes and this is thought to be the first report of such evidence; it is of particular relevance as it increases evidence for a possible saprophytic mode of growth. This supported the activities determined under *in vitro* conditions and suggested that such conditions mimicked those found *in situ*. The detection of endo-β-1,4-glucanase, xylanase, chitinase and laminarinase indicate that the secretion of the depolymerases is necessary for survival in the compost environment. Sterile compost was used to remove potential contaminants that may have contributed to the enzymic activity detected within the compost. This process was very difficult as non-sterile compost harbours many thermophiles that become activated by the heat sterilisation (see section 5.1.4). Even after an intense sterilisation procedure, some low enzyme activity from contaminants was detected. However the Nelson-Somoygi sugar determination method used is highly sensitive and the activities detected were of much lower magnitude than those detected here *in vitro* in the presence of wheat straw (typically 10.1-fold and 2.9-fold for cellulase and xylanase respectively). Also substrate-containing overlays failed to detect activity in the compost controls confirming that contaminant activity was very low. This suggested that enhanced activities found in *T. harzianum*-infested compost and the detection of isoforms on substrate overlays was demonstrative of extracellular enzyme activity of *T. harzianum* involved in the colonisation of sterile compost.

Extracellular enzyme activities of *T. harzianum* from sterile compost after 2 weeks incubation was higher from the non-aggressive rather than from the aggressive genotypes. Differences in enzyme activities may reflect the level or type of *T. harzianum* growth since Th1 sporulated abundantly but had limited mycelial growth. The enzyme activities shown were detected after 2 weeks incubation but samples removed after 1 and 3 weeks exhibited quite different activities (see Appendix B.2) and some later samples revealed higher extracellular activities from aggressive genotypes, particularly Th2, than from non-aggressive genotypes.

In sterile compost, *T. harzianum* genotypes may also degrade the lysed bacterial cells and chitinase activity could also be indicative of lysozyme activity (R. Loewe, Loewe Biochemica GMbH, pers. com.). Lysozyme selectively degrades the peptidoglycan component of Gram-positive bacterial cell walls (Pelczar *et al.* 1986). Peptidoglycan, a large polymer, consists of sugars (N-acetylglucosamine and N-acetylmuramic acid) and amino acids; N-acetylglucosamine is the monomeric form of chitin and explains why some lysozymes and chitinases show cross reactivity (Terwisscha van Scheltinga *et al.* 1995).

Isoforms of laminarinase, endo-β-1,4-glucanase and endo-β-1,4-xylanase were detected from all *T. harzianum* genotypes in sterile compost (Table 5.7). The non-aggressive genotypes, particularly Th3, produced more isoforms than aggressive genotypes. An endo-β-1,4-glucanase isoform of pI 4.69 was secreted by all genotypes and a similar isoform (pI 4.78) was produced by all genotypes in the presence of wheat straw, *in vitro*. There were no endo-β-1,4-glucanase isoforms that distinguished the aggressive from the non-aggressive genotypes. The failure to detect in compost any of the isoforms that appeared to be specific to aggressive genotypes *in vitro* may have been due to the selection of one time sample for IEF (limited due to expense). It is possible that some of the genotype-specific isoforms were produced at a later stage in colonisation as suggested by the increase in cellulase activity for Th2 after three weeks (see Appendix B.2).

Th1 and Th3 produced more xylanase isoforms than did Th2 and Th4. However Th2 and Th4 produced more xylanase isoforms than cellulase. Hemicellulases may be important for the degradation of rye grain (the inoculum base and initial nutrient source) which contains high levels of hemicellulose, *ca.* 75% of grain cell wall dry weight (Kent 1983) and this

may explain the high xylanase activity of *T. harzianum* genotypes. The isoelectric points of xylanase isoforms produced in sterile compost displayed considerable similarity with isoforms secreted in the presence of wheat straw, *in vitro* (Table 5.7). Likewise Calonje *et al.* (1997) found that depolymerase activities produced *in vitro* by *V. fungicola* in the presence of *A. bisporus* cell walls were also detected *in vivo*.

Laminarinase existed as more isoforms in sterile compost than in the presence of *A. bisporus* cell walls or wheat straw (*in vitro*). Th2 displayed isoforms identical to those of Th4 (pI 5.67 and 5.99), that were not detected from Th2 in the presence of wheat straw (*in vitro*). Therefore Th2 exhibited laminarinase activity *in situ* that was characteristic of Th4. This activity may be induced when Th2 entered a potential infection cycle as opposed to *in vitro* culture conditions. Although not clearly visible from the gel image (Fig 5.17c) the differences were clear following immediate measurements on a powerful light box. All genotypes displayed more complex laminarinase isoform profiles *in situ* compared to the *in vitro* culture conditions. The laminarinase isoforms detected *in vitro* could be representative of constitutive activity (de la Cruz *et al.* 1993).

Notably, Th3 differed not only from the aggressive genotypes but also from the other non-aggressive Th1 throughout the studies on depolymerase isoforms. This difference was detected both *in vitro* and *in vivo* and therefore confirms the re-classification of Th3 to *T. atroviride* (Muthumeenakshi *et al.* 1998).

5.2.4 Development of a PCR-based diagnostic test for aggressive T. harzianum genotypes

The molecular techniques previously used to determine the biotype of *T. harzianum* strains require considerable experience and expertise. The time factor involved is also critical when dealing with a rapidly disseminating and devastating disease like *T. harzianum* green mould. The development of a simple, positive/negative diagnostic test is essential.

The initial application of *cel1* primers to genomic DNA from *T. harzianum* strains produced amplicons that appeared to distinguish aggressive from non-aggressive genotypes. Furthermore, the *T. harzianum* genotype amplicons were distinguished from the *A. bisporus* amplicon. The primers appeared to be selective for *A. bisporus* and *T. harzianum* after a preliminary test with four other species, however the test should be

Table 5.7 Isoforms of depolymerases secreted by *T. harzianum* genotypes in sterile compost.

Depolymerase	*T. harzianum* genotype	Isoelectric point	*In vitro* equivalent*
endo-β-1,4-glucanase	Th1	4.69, 6.97, 7.30	4.78, 6.44, 7.78
	Th3	4.43 to 8.99	3.96 to 7.02 (4.78)
	Th2	4.69	4.78
	Th4	4.69	4.78
endo-β-1,4-xylanase	Th1	6.25, 6.91, 7.36, 8.92, 9.25, 9.51	8.80, 9.24
	Th3	4.76 to 9.38 (6.25, 6.91, 9.25)	4.66, 4.92, 5.3, 5.55, 6.0, 9.24, 9.37
	Th2	4.95, 6.25, 6.91, 9.06	5.04, 5.74, 8.80
	Th4	6.25, 6.91, 7.36, 8.92, 9.12	5.74, 8.80
laminarinase	Th1	4.95, 5.15 7.23 to 8.08	4.98
	Th3	4.95, 5.15 6.51 to 8.21	4.98
	Th2	4.95, 5.15, 5.67, 5.8, 5.99	4.98
	Th4	4.95, 5.15, 5.67, 5.99, 6.25, 6.58	4.98, 6.19 6.19

* Possible equivalent isoforms of depolymerase produced, *in vitro*, in the presence of wheat straw.

extended to include species of fungi and bacteria isolated from mushroom compost. The size difference in the amplicons produced by different genotypes of *T. harzianum* were substantial (*ca.* 400 bp and 700 bp for Th1 and aggressive genotypes, respectively) which suggested visible assessment of the *T. harzianum* genotype would be simple.

This initial determination of genotypic differences at the molecular level appeared to be the basis of a robust diagnostic test. However, when the study was extended to include more strains of *T. harzianum* some variation was detected within some of the genotypes. Th4 exhibited no variation and each of three strains produced the 700 bp amplicon. Three strains of Th1 were used of which only one strain (Th1(c)) produced an amplicon. The failure to amplify a product from some Th1 strains was not a problem since the test should detect the presence of aggressive strains of *T. harzianum*. Th3 strains exhibited two low yielding products which were consistent for the three strains assessed. While the smaller of the products was identical to the 700 bp product amplified from aggressive genotype DNA, the amplification of a larger product (*ca.* 850 bp) allowed the distinction of these strains from aggressive strains. The variation displayed by Th2 was discouraging in terms of producing a robust diagnostic test based on this set of primers. Three of the six Th2 strains tested produced an amplicon very similar in size, perhaps slightly smaller, to that of Th1. Although two of these strains also produced the 700 bp fragment, one strain did not and only produced the small fragment. Similar amplicons produced by different genotypes would make an assessment for aggressive strains very difficult after PCR and gel electrophoresis alone and any additional assessment, such as amplicon sequence determination, would require more technical experience. Therefore at the outset this particular set of primers could not be recommended for further development of a diagnostic test, however more strains should be tested before the potential test is discarded.

The amplicons from Th1 (Th1(c)), Th2 (T7) and Th4 (RM10c) were sequenced. *T. harzianum* amplicon sequences exhibited no homology with the *A. bisporus* amplicon or to any other sequence on the database, suggesting random amplification from *T. harzianum* genomic DNA. The sequences of products characteristic of aggressive genotypes displayed high homology, while the Th1 amplicon had no homology to the 700 bp product from aggressive genotypes. The oligonucleotides appeared to hybridise to a different region in the genome of the non-aggressive strain. A study of the products from other strains would be necessary to determine if this difference is consistent. Furthermore it would be

interesting to compare these sequences with those of Th3 products and the additional products of some of the Th2 strains. In summary, although this test was able to distinguish most strains of *T. harzianum* tested according to their genotypes there were some strains which produced amplicons very similar to other genotypes, making accurate assessments impossible. To identify conclusively aggressive *T. harzianum* genotypes from a field sample, a simple test should give a visible positive/negative result. For example a test has been reported that detects a potential green mould threat by the production of a 444 bp amplicon which is unique to Th2 and Th4 (Chen *et al.* 1999).

To summarise, the research in this section has concentrated on the saprophytic abilities of *T. harzianum* in mushroom compost. This is the first report of the enhanced saprophytic growth of Th2 and Th4 in compost, which could have an essential role in the development of *T. harzianum* infestations of mushroom compost. Depolymerase activities that could be associated with saprophytic growth have been detected *in vitro* and *in situ*. The retrieval of enzyme activity from compost inoculated with *T. harzianum* genotypes is also thought to be the first report of this nature in this particular system. Depolymerase isoforms from sterile compost displayed similarities with those detected *in vitro*. However laminarinase isoform profiles from compost differed to those found *in vitro* suggesting that different isoforms were triggered *in situ*. In addition Th2 displayed isoforms similar to Th4 only in sterile compost. This evidence overall points towards saprotrophy as the key mechanism for the antagonist *T. harzianum* in mushroom compost.

Chapter Six

General Discussion

The research described in this thesis concerns the nature of the possible interaction(s) between aggressive compost-colonising *T. harzianum* genotypes and *A. bisporus*. The study has focussed on the depolymerases produced by *T. harzianum* necessary for a putative mycoparasitic attack towards *A. bisporus* and those enzymes capable of degrading available nutrients for a saprophytic existence. An enhanced saprophytic ability has been revealed in aggressive *T. harzianum* genotypes and this is thought to be the first report of such behaviour linked to *T. harzianum* green mould in mushroom compost. It is suggested here that a saprophytic growth stage may be a prerequisite for *T. harzianum* infestations in mushroom compost.

Investigations of interactions on nutrient agar media and in compost revealed evidence of competition between *T. harzianum* and *A. bisporus*, which may involve non-volatile and volatile substances, produced by both fungi. *A. bisporus* inhibited *T. harzianum* at close proximity when separated physically, suggesting the involvement of short-range volatile antibiotics. Aggressive *T. harzianum* only affected *A. bisporus* when in contact and this was indicative of a possible mycoparasitic stage or a highly competitive stage in which *T. harzianum* produced putative non-volatile antibiotics and/or actively scavenged the degradation products of *A. bisporus*.

Several depolymerase isoforms were linked to *T. harzianum* aggressiveness and these included endo-β-1,4-glucanase, chitinase and protease. The fibrillar components of cereal and *A. bisporus* cell walls appeared to trigger unique depolymerase isoforms of glucanase and chitinase in Th2 and Th4. Notably all *T. harzianum* genotypes produced similar laminarinase and xylanase isoforms *in vitro*. Since xylan and β-1,3-glucan comprise the matrix component of cereal and fungal cell walls respectively, perhaps activities to degrade the matrix of cell walls are of less importance for aggressive colonisation of mushroom compost. Chitinases have important roles in the parasitism of *S. rolfsii* by *T. harzianum* (Inbar and Chet 1995). Anomalously, this pattern would contrast with that exhibited by plant pathogens for which enzyme activities corresponding to the host cell wall matrix polymers play the key role (Cooper 1984; Cooper *et al.* 1988). However a protease (pI

6.22) apparently linked to aggressive *T. harzianum* may have a role in mycoparasitism where its activity would degrade proteinaceous element of the fungal cell wall matrix.

There was no evidence to suggest that the interaction between aggressive *T. harzianum* genotypes and *A. bisporus* was exclusively mycoparasitic and this supported the observations of Fletcher (1997). In contrast to other systems, in which *T. harzianum* is indisputably an invasive mycoparasite (Elad *et al.* 1983a; Benhamou and Chet 1996), the evidence here indicates a complex interaction involving several types of antagonism, including competition and possibly infrequent mycoparasitism. The discovery of the saprophytic capabilities of aggressive *T. harzianum* (with the possibility of additional antagonistic action) suggests that the term 'competitive saprophytic ability' (CSA) described by Garrett (1970) may be applicable. Garrett (1970) described a high CSA with the following list:

1. Rapid rate of spore germination in response to the presence of nutrients.
2. Rapid rate of growth.
3. Efficient production of depolymerases, or production of unique depolymerases.
4. Tolerance of environmental extremes, including extremes of nutrient concentration.
5. Ability to utilize available nutrients.
6. Parasitism or other forms of antagonism.
7. Tolerance of antibiotics or other forms of antagonism.

Aggressive *T. harzianum* genotypes meet the criteria listed above with the exception of a delayed sporulation rate (*cf.* non-aggressive) (Seaby 1987). The CSA of *A. bisporus* would appear to be lower than Th2 and Th4 since spore germination and growth rates are slow, being K-selected (Pianka 1970). *A. bisporus* has adapted to a relatively constant environment and therefore may be intolerant of environmental extremes. In addition the author found no evidence of parasitism of *T. harzianum* by *A. bisporus*; however the production of inhibitory substances could be indicative of antibiosis.

The level of CSA may also be used to compare the genotypes of *T. harzianum*. While aggressive genotypes appear to have a high CSA, a lower CSA may be exhibited by non-aggressive genotypes (Th1 and Th3). Th1 is apparently a ruderal fungus that directs energy into reproduction in order to disseminate spores to new environments. The low CSA of Th1 might be determined by the lack of unique depolymerases and other forms of antagonism as well as its low tolerance to *A. bisporus* antibiotic compounds and a slow

growth rate in compost. Th3 may exhibit a higher CSA than Th1 since it demonstrated enhanced saprophytic ability and production of some unique depolymerase isoforms. However Th3 did not exhibit other forms of antagonism and therefore this distinguished it from the aggressive genotypes.

Ratto *et al.* (1996) reported that *T. harzianum* was capable of iron chelation by the production of siderophores. Furthermore, Altomare *et al.* (1999) described the ability of *T. harzianum* to dissolve insoluble phosphates and micronutrients *via* chelation and redox mechanisms. These reports support the suggestion that *T. harzianum* may be an effective scavenger of the available nutrients and the degradation products of *A. bisporus*. Altomare *et al.* (1999) did not detect oxalic acid or any other organic acid in culture fluids and therefore calcium and phosphate solubilization and chelation occurred by a different mechanism to that of *A. bisporus* (Dutton *et al.* 1993). Th2 and Th4 demonstrated higher phosphatase activity than did Th1 and Th3 in the presence of *A. bisporus* cell wall extract.

The switch from competitive interactions to parasitism may occur rapidly for *T. harzianum* since it is incapable of degrading the lignin complex present in mushroom compost and therefore may experience conditions of nutrient stress before *A. bisporus*. However this putative switch in type of antagonism may also be associated with the growth stage of *A. bisporus*. Mycelial growth of *A. bisporus* is distinctive due to the presence of copious calcium-rich crystals, which may prevent close contact between *A. bisporus* and *T. harzianum*. Masaphy *et al.* (1987) reported that the crystals were not present on reproductive strands and suggested that crystals would interfere with strand formation. Therefore it is suggested here that the early stages of reproductive mycelia may be vulnerable to a mycoparasitic attack in the absence of calcium-rich crystals and it may be at this stage that the interaction switches from competition to parasitism. Sharma *et al.* (1999) reported that Th2 colonisation rapidly increased with the first flush of mushrooms and continued to increase as cropping progressed. However as the observations in this study occurred during vegetative colonisation by *A. bisporus* this may explain the infrequency of mycoparasitism.

The evidence from this research points towards a competitive interaction between *T. harzianum* and *A. bisporus* in which the enhanced saprophytic ability of aggressive *T. harzianum* is essential for green mould infestations. The production by *T. harzianum* of

cell wall-degrading enzymes potentially involved in mycoparasitism as well as the secretion of antibiotic substances by *T. harzianum* may also be important for successful colonisation.

Future Work

Sharma *et al.* (1999) reported that Th2 was affected by the same compost characteristics important for compost quality such as moisture content, conductivity, ash and extent of fermentation. The level of Th2 colonisation was higher in the superior quality compost compared to another sample in which components were not optimal for mushroom production (Sharma *et al.* 1999). This suggests that Th2 and Th4 have evolved survival strategies for this niche rather than only infesting poor quality compost. In this work high quality mushroom compost (supplied by HRI Wellesbourne) from many different batches was used throughout the experimentation but development of aggressive *T. harzianum* infestations were consistent and this supports the findings of Sharma *et al.* (1999). Therefore stringent hygiene practices will reduce incidents but will probably not completely eradicate *T. harzianum* green mould. Further understanding of the biochemistry of the antagonism by *T. harzianum* will be required.

An obvious continuation of this work would be to consider the endo-β-1,4-glucanase, chitinase and protease isoforms specific to aggressive genotypes. The isoform profile produced by *T. harzianum* genotypes in compost should be investigated at various stages of colonisation since preliminary studies suggested differences between the genotypes and changes in enzyme activities during colonisation. Isoforms specific to aggressive *T. harzianum* in compost could lead to characterisation of the relevant genes and this would allow sequence comparison with similar depolymerases important in other *T. harzianum* systems. Targeted disruption of relevant genes may reveal whether these activities are essential for compost colonisation or if the activity is simply contributory to the overall success of the fungus. Woo *et al.* (1999) confirmed a mycoparasitic role for endochitinase (CHIT42) by disruption of the gene *ech42* which subsequently resulted in a 40% reduction in antifungal activity of *T. harzianum* P1.

It was suggested from this work, that enhanced saprophytic growth could be essential for *T. harzianum* infestations caused by Th2 and Th4. To substantiate this proposal it would be

interesting to remove this ability from the aggressive genotypes or up-regulate it and determine the consequences. The association of several endo-β-1,4-glucanase isoforms with to aggressive behaviour may be of importance for the saprophytic growth in compost and these enzymes could be purified and characterised leading to subsequent molecular characterisation of the genes. Inactivation of these genes may reveal impairment to the aggressive behaviour of *T. harzianum*. Likewise, non-aggressive *T. harzianum* strains could be transformed with aggressive-specific genes and their effect(s) on compost colonisation followed.

The discovery of a protease isoform specific to aggressive behaviour in *T. harzianum* may be important since the protease prb1 has an essential role in the mycoparasitism of *R. solani* by *T. harzianum* (Flores *et al.* 1997). Th2 and Th4 failed to exhibit frequent or exclusive mycoparasitism but a few incidents were recorded and therefore it is possible that parasitism of *A. bisporus* may occur. Molecular characterisation of the protease isoform with pI 6.22 would allow sequence comparison with prb1. Transformation of aggressive *T. harzianum* to over-express protease pI 6.22 would be necessary to determine a potential role in a mycoparasitic interaction which may be observed as an increase in detrimental effects towards *A. bisporus*. In addition the application of protease inhibitors specific to protease pI 6.22 could be used to assess the effect of this activity on the growth of *A. bisporus* in *T. harzianum* infested compost.

The enhanced saprophytic growth of *T. harzianum* genotypes 2, 3 and 4 in non-sterile compost was revealed by quantification of *T. harzianum* cfu. The same technique could be used to quantify *T. harzianum* in the presence of *A. bisporus*, while the biomass of *A. bisporus* could be determined by quantification of the laccase activity (Turner *et al.* 1975; Wood 1980). These techniques would provide a more accurate record of the growth of *T. harzianum* and *A. bisporus* than the visual scales used here in preliminary work. The changes in biomass could be followed during colonisation to reveal at which stage aggressive *T. harzianum* affects the growth of *A. bisporus* and to give a more accurate comparison of relative aggressiveness of *T. harzianum* isolates.

It could be valuable to investigate the chelating abilities of Th2 and Th4 for various essential nutrients such as phosphates and iron. Comparisons between *T. harzianum*

genotypes may reveal another genotype-distinguishing feature. Investigating the relative efficacy of chelation and the mechanisms involved for aggressive *T. harzianum* and *A. bisporus* may reveal another aspect to a competitive interaction.

This research has continued to reveal the complex nature of the interactions between aggressive *T. harzianum* genotypes and *A. bisporus*. The evidence indicates a competitive mechanism involving saprophytic abilities and more than one form of antagonism. The work here has also exposed several new areas for future work that will be necessary for a comprehensive understanding of the mechanisms of antagonism.

References

Abercrombie J. 1779. In: The garden mushroom, its nature and cultivation. Lockyer Davis, London.

Ahmad JS and Baker R. 1987. Competitive saprophytic ability and cellulolytic ability of rhizosphere-competent mutants of *Trichoderma harzianum*. Phytopathology **77**: 358-362.

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD. 1989. Molecular biology of the cell. Second edition. Garland Publishing Inc, New York and London.

Altomare C, Norvell WA, Bjorkman T and Harman GE. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Appl Environ Microbiol **65**(7):2926-2933.

Alurralde JL and Ellenrieder G. 1984. Effect of attached carbohydrates on the activity of *Trichoderma viride* cellulases. Enzyme Microbiol Tech **6**:467-470.

Andrews JH and Harris RF. 1997. 1. Dormancy, Germination, Growth, Sporulation and Dispersal. In: The Mycota IV: Environmental and Microbial Relationships. Eds. D.T. Wicklow and B. Soderstrom. Springer-Verlag Berlin Heidelberg.

Ann PJ and Ko WH. 1990. Survey of enzyme activity on solid media in *Phytophthora*. Can. J. Bot. **68**: 139-143.

Arras G and Arru S. 1997. Mechanisms of action of some microbial antagonists against fungal pathogens. Annali di Microbiologia ed Enzimologia **47**: 97-120.

Askew DJ and Laing MD. 1993. An adapted selective medium for the quantitative isolation of *Trichoderma* species. Plant Pathology **42**: 686-690.

Atkey PT. 1985. 14. Viruses. In: Flegg PB, Spencer DM, Wood DA (eds) The biology and technology of the cultivated mushroom. John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore.

Atkey PT and Wood DA. 1983. An electron microscope study of wheat straw composted as a substrate for the cultivation of the edible mushroom (*Agaricus bisporus*). J Appl Bacteriol **55**:293-304.

Badham ER. 1991. Growth and competition between *Lentinus edodes* and *Trichoderma harzianum* on sawdust substrates. Mycologia **84**(4): 455-463.

Baker R. 1987. Mycoparasitism: Ecology and Physiology. Canadian J. Plant Pathology **9**: 370-379.

Baker and Griffin. 1995. Molecular strategies for biological control of fungal plant pathogens. In: Reuveni R (ed) Novel approaches to integrated pest management. Lewis, CRC Press, Boca Raton.

Barak R, Elad Y, Mirelman D and Chet I. 1985. Lectins: a possible basis for specific recognition in the interaction of *Trichoderma* and *Sclerotium rolfsii*. Phytopathol **77**:458-462.

Barak R, Elad Y and Chet I. 1986. The properties of L-fucose binding agglutinin associated with the cell wall of *Rhizoctonia solani.* Arch Microbiol **144**:346-349.

Bartnicki-Garcia S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Ann Rev Microbiol **22**:87-108.

Belanger RR, Dufour N, Caron J and Benhamou N. 1995. Chronological events associated with the antagonistic properties of *Trichoderma harzianum* against *Botrytis cinerea*: indirect evidence for sequential role of antibiosis and parasitism. Biocontrol Science and Technology **5**: 41-53.

Benhamou N and Chet I. 1993. Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology **83**(10): 1062-1071.

Benhamou N and Chet I. 1996. Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*: ultrastructural and cytochemical aspects of the interaction. Phytopathology **86**(4): 405-416.

Benhamou N and Chet I. 1997. Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and *Pythium ultimum.* Applied and Environmental Microbiology **63**(5): 2095-2099.

Beynon RJ and Salvesen G. 1989. Appendix III: Commercially available protease inhibitors. In: Practical Approach: Proteolytic Enzymes. Eds. R.J. Beynon and J.S. Bond. IRL Press.

Biely P, Mislovicova D and Toman R. 1985a. Soluble chromogenic substrates for the assay of endo-1,4-β-xylanases. Analytical Biochemistry **144**: 142-146.

Biely P, Markovic O and Mislovicova D. 1985b. Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. Analytical Biochemistry **144**: 147-151.

Biely P, Kremnicky L, Alfold J and Tenkanen M. 1994. Stereochemistry of the hydrolysis of glycosidic linkage by endo-1,4-β-xylanases of *Trichoderma reesei.*FEB Letters **356**:137-140.

Biely P, Vrsanska M, Tenkanen M and Kluepfel D. 1997. Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnology **57**:151-166.

Bohus G. 1959. Investigations concerning the life processes of the cultivated mushroom. Mushroom Science **4**:86-131.

Brockmann B, Smit R and Tudzynski P. 1992. Characterization of an extracellular β-1,3-glucanase of *Claviceps purpurea.* Physiological and Molecular Plant Pathology **40**: 191-201.

Burnett JH and Trinci APJ (eds). 1979. Fungal walls and hyphal growth. Cambridge University Press, London, New York, Melbourne.

Burton, K.S., J.F. Smith, D.A. Wood and C.F. Thurston. 1997. Extracellular proteinases from the mycelium of the cultivated mushroom *Agaricus bisporus*. Mycological Research **101**(11): 1341-1347.

Calonje M, Garcia Mendoza C and Perez Cabo A. 1995. Some significant differences in wall chemistry among four commercial *Agaricus bisporus* strains. Current Microbiology **30**: 111-115.

Calonje M, Garcia Mendoza C, Galan B and Novaes-Ledieu M. 1997. Enzymic activity of the mycoparasite *Verticillium fungicola* on *Agaricus bisporus* fruit body cell walls. Microbiology **143**: 2999-3006.

Campbell LL, Williams J and OB. 1951. A study of chitin-decomposing micro-organisms of marine origin. J Gen Microbiol **5**: 894-905.

Campbell RL, Rose DR, Wakarchuk WW, To R, Sung W and Yaguchi M. 1993. A comparison of the structures of the 20 kd xylanases from *Trichoderma harzianum* and *Bacillus circulans*. Proceedings of 2nd TRICEL symposoium on *Trichoderma reesei* cellulases and other hydrolases. Foundation for biotechnical and industrial fermentation research **8**:63-72.

Carlile AJ, Clarkson JM and Cooper RM. 1999. Purification and characterisation of an alkaline trypsin-like protease secreted by *Stagonospora nodorum* in culture and in infected wheat. MPMI submitted.

Carpita NC. 1996. Structure and biogenesis of the cell walls of grasses. Ann Rev Plant Physiol Plant Mol Biol **47**:445-476.

Carpita NC and Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. The Plant Journal **3**(1): 1-30.

Carsolio C, Gutierrez A, Jimenez B, Van Montagu M and Herrera-Estrella A. 1994. Characterisation of *ech-42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA **91**: 10903-10907.

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I and Herrera-Estrella A. 1999. Role of the *Trichoderma harzianum* endochitinase gene *ech42*, in mycoparasitism. Appl Env Microbiol **65**(3):929-935.

Castle A, Speranzini D, Rghei N, Alm G, Rinker D and Bisset J. 1998. Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Appl and Env Microbiol **64**(1):133-137.

Cazemier AE, Hackstein JHP, Op den Camp HJM, Rosenberg J and Van Der Drift C. 1997. Bacteria in the intestinal tract of different species of arthropod. Microbial Ecology **33**: 189-197.

Chang ST and Hayes WA (eds). 1978. The biology and cultivation of edible mushrooms. Academic Press, Inc., London.

Chang TT, Yang XY and Ko WH. 1992. A sensitive method for detecting production of extracellular enzymes by fungi on solid media. Mycologia **84**(6): 923-926.

Chang Y. 1967. The fungi of wheat straw compost.II. Biochemical and physiological studies. Trans Br Mycol Soc **50**(4):667-677.

Chang Y and Hudson HJ. 1967. The fungi of wheat straw compost. I. Ecological studies. Trans Br Mycol Soc **50**(4):649-666.

Chen X, Ospina-Giraldo M, Tan Q, Schlagnhaufer B, Royse DJ and Romaine CP. 1997. Molecular genetic analysis of *Trichoderma* associated with mushroom green mold in Pennsylvania. Phytopathology **87**:S18 publication no. P-1997-0125-AMA.

Chen X, Romaine CP, Ospina-Giraldo MD and Royse DJ. 1999. A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. Appl Microbiol Biotech **52**(2):246-250.

Cherif M and Benhamou N. 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology **80**: 1406-1414.

Chet I. 1987. 6. *Trichoderma* – application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: Innovative Approaches to Plant Disease Control. I.Chet (Ed.). J. Wiley and Sons (Publ.).

Chet I, Harman GE and Baker R. 1981. *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. Microbial Ecology **7**:29-39.

Chet I, Inbar J and Hadar Y. 1997. 11.Fungal antagonists and mycoparasites. In: Wicklow DT, Soderstrom B (eds) The Mycota IV: Environmental and Microbial Relationships, Springer-Verlag, Berlin, Heidelberg, pp.165-184.

Claydon N, Allan M and Wood DA. 1988. Fruit body biomass regulated production of extracellular endocellulase during periodic fruiting by *Agaricus bisporus*. Trans. Br. Mycol. Soc. **90**(1): 85-90.

Claydon N, Allan M, Hanson JR and Avent AG. 1987. Antifungal alky pyrones of *Trichoderma harzianum* (Rifai). Trans. Br. Mycol. Soc. **88**: 503-513.

Cobb BD and Clarkson JM. 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nucl. Acids Res. **22**(18):3801-3805.

Cobb BD. 1996. Strategies for carbon metabolism by the entomopathogenic fungus *Metarhizium anisopliae*. PhD Thesis, University of Bath.

Cooke RC and Rayner ADM. 1984. Ecology of Saprotrophic Fungi. Longman, London and New York.

Cooper RM, Longman D, Campbell A, Henry M and Lees PE. 1988. Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. Physiol Molec Plant Pathol **32**:33-47.

Cooper RM. 1984. The role of cell wall-degrading enzymes in infection and damage. In: Plant Diseases: infection, damage and loss (eds) Wood RKS and Jellis GJ. Blackwell Scientific Publications, Oxford pp. 13-27.

Cooper RM and Wood RKS. 1975. Regulation of synthesis of cell wall-degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Pathol **5**:135-156.

Cooper RM and Wood RKS. 1980. Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endo-pectin lyase in *Verticillium* wilt of tomato. Physiol Plant Pathol **16**:285-300.

Cooper RM, Longman D, Campbell A, Henry M and Lees PE. 1988. Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. Physiol Molec Plant Pathol **32**:33-47.

Coughlan, M.P. 1988. Staining techniques for the detection of the individual components of cellulolytic enzyme systems. Methods in Enzymology **160**: 135-144.

Craig GD, Gull K and Wood DA. 1977. Stipe elongation in *Agaricus bisporus*. J Gen Microbiol **102**:337-347.

Cronin DA and Ward MK. 1971. The characterization of some mushroom volatiles. J Sci Food Agric **22**:477-479.

Cruz C, Noel-Suberville C and Montury M. 1997. Fatty acid content and some flavour compound release in two strains of *Agaricus bisporus*, according to three stages of development. J. Agric. Food Chem. **45**: 64-67.

Dal Soglio FK, Bertagnolli BL, Sinclair JB, Yu G-Y and Eastburn DM. 1998. Production of chitinolytic enzymes and endo-glucanase in the soybean rhizosphere in the presence of *Trichoderma harzianum* and *Rhizoctonia solani*. Biological Control **12**:111-117.

De la Cruz J, Hidalgo-Gallego A, Benitez T, Pintor-Toro JA and Llobell A. 1992. Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur J Biochem **206**:859-867.

De la Cruz J, Rey M, Lora JM, Hidalgo-Gallego A, Dominguez F, Pintor-Toro JA, Llobell A and Benitez T. 1993. Carbon source control on β-glucanases, chitobiase and chitinase from *Trichoderma harzianum*. Arch. Microbiol. **159**: 316-322.

De la Cruz J, Pintor-Toro JA, Benitez T, Llobell A and Romero LC. 1995a. A novel endo-β-1,3-glucanase, BGN13.I, involved in the mycoparasitism of *Trichoderma harzianum*. J. Bacteriology **177**(23): 6937-6945.

De la Cruz J, Pintor-Toro JA, Benitez T and Llobell A. 1995b. Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriology **177**: 1864-1871.

Deacon JW. 1984. Introduction to Modern Mycology 2nd Edition. Blackwell Scientific Publications, Oxford.

Deane EE, Whipps JM, Lynch JM and Peberdy JF. 1998. The purification and characterization of a *Trichoderma harzianum* exochitinase. Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology **1383**:101-110.

Dennis C and Webster J. 1971a. Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. **57**(1):25-39.

Dennis C and Webster J. 1971b. Antagonistic properties of species-groups of *Trichoderma.* II. Production of volatile antibiotics. Trans. Br. Mycol. Soc. **57**(1):41-48.

Dennis C and Webster J. 1971c. Antagonistic properties of species-groups of *Trichoderma.* III. Hyphal interaction. Trans Br mycol Soc **57**(3):363-369.

Deverall BJ. 1981. Fungal Parasitism 2nd Edition. Camelot Press Ltd, Great Britain.

Di Pietro A, Lorito M, Hayes CK, Broadway RM and Harman GE. 1993. Endochitinase from *Gliocladium virens:* Isolation, characterization and synergistic antifungal activity n combination with gliotoxin. Phytopathology **83**:308-313.

Dragt JW, Geels FP, De Bruijn WC and Van Griensven LJLD. 1996. Intracellular infection of the cultivated mushroom *Agaricus bisporus* by the mycoparasite *Verticillium fungicola.* Mycological Research **100**(9):1082-1086.

Dubos B. 1984. Biocontrol of *Botrytis cinerea* on grapevines by an antagonistic strain of *Trichoderma harzianum.* In: Current Perspectives in Microbial Ecology (Proceedings of the Third International Symposium on Microbial Ecology, Michigan State University), M.J. Klug and C.A. Reddy (eds), American Society for Microbiology, Washington, D.C. pp 370-373.

Durrant AJ, Wood DA and Cain RB. 1991. Lignocellulose biodegradation by *Agaricus bisporus* during solid substrate fermentation. J Gen Microbiol **137**:751-755.

Dutton MV, Evans CS, Atkey PT and Wood DA. 1993. Oxalate production by Basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium.* Applied Microbiology and Biotechnology **39**:5-10.

Elad Y. 1995. Interaction of *Trichoderma* and *Gliocladium* spp. with host fungi. In: Pathogenesis and host specificity in plant diseases. Histopathological, biochemical, genetic and molecular bases. Volume II Eurkaryotes. K. Kohmoto, U.S. Singh, R.P. Singh (eds), Pergamon pp291-293.

Elad Y, Chet I and Katan J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani.* Phytopathol **70**(2):119-121.

Elad Y, Chet I and Henis Y. 1981. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica **9**(1):59-67.

Elad Y, Chet I and Henis Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian J. Microbiol. **28**:719-725.

Elad Y, Barak R, Chet I and Henis Y. 1983a. Ultrastructural studies of the interaction between *Trichoderma* spp. and plant pathogenic fungi. Phytopathologische Zeitschrift **107**:168-175.

Elad Y, Barak R and Chet I. 1983b. Possible roles of lectins in mycoparasitism. J Bacteriol **154**:1431-1435.

Elad Y, Barak R and Chet I. 1984. Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*. Soil Biol Biochem **16**:381-386.

Elad Y, Kohl J and Fokkema NJ. 1994. Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic yeasts. Phytopathology **84**:1193-1200.

Elliot T. 1997. Mushrooms. SGM Quarterly February 1997. pp.8-9.

Emmett RW and Parbery DG. 1975. Appressoria. Annu Rev Phytopathol **13**:147-167.

Evered CE, Noble R and Atkey PT. 1995. Microbial populations and straw degradation in mushroom composts prepared in controlled environments. In: Elliot TJ (ed) Science and cultivation of edible fungi, volume 1, Balkema, Rotterdam. pp.245-250.

Farkas V, Liskova M and Biely P. 1985. Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiology Letters **28**:137-140.

Fergus CL. 1964. Thermophilic and thermotolerant moulds and actinomycetes of mushroom compost during peak-heating. Mycologia **56**:267-284.

Fermor TR and Wood DA. 1981. Degradation of bacteria by *Agaricus bisporus* and other fungi. J Gen Microbiol **126**:377-387.

Fermor TR and Grant WD. 1985a. Degradation of fungal and actinomycete mycelia by *Agaricus bisporus*. J Gen Microbiol **131**:1729-1734.

Fermor TR, Smith JF and Spencer DM. 1979. The microflora of experimental mushroom composts. J Hort Sci **54**(2):137-147.

Fermor TR, Randle PE and Smith JF. 1985b. Compost as a substrate and its preparation. In: Flegg PB, Spencer DM, wood DA (eds) The biology and technology of the cultivated mushroom, John Wiley & Sons Ltd. pp.81-109.

Fermor TR, Wood DA, Lincoln SP and Fenlon JS. 1991. Bacteriolysis of *Agaricus bisporus*. J Gen Microbiol **137**:15-22.

Flegg PB and Wood DA. 1985. Growth and fruiting. In: Flegg PB, Spencer DM, Wood DA (eds) The biology and technology of the cultivated mushroom. John Wiley and Sons Ltd, Dorchester. pp.141-177.

Fletcher JT, White PF and Gaze RH. 1989. Mushrooms: Pests and Disease Control 2nd Edition. Intercept, Andover, Hants.

Fletcher JT. 1997. Mushroom spawn and the development of *Trichoderma* compost mold. Mushroom News **45**(8):6-11.

Flores A, Chet I and Herrera-Estrella A. 1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb1*. Curr. Genet. **31**:30-37.

Fravel DR. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol. **26**:75-91.

Gama FM, Villanova M and Mota M. 1998. Exo- and Endo-glucanolytic activity of cellulases purified from *Trichoderma reesei*. Biotechnology Techniques **12**(9):677-681.

Gandy DG. 1985. Bacterial and Fungal Diseases. In: Flegg PB, Spencer DM and Wood DA (eds) Cell wall structure and systhesis (*A.bisporus*). In: The biology and technology of the cultivated mushroom. John Wiley and Sons Ltd, Dorchester.

Garcia Mendoza C, Avellan MA, Sanchez E and Novaes-Ledieu M. 1987. Differentiation and wall chemistry and *Agaricus bisporus* vegetative and aggregated mycelia. Arch. Microbiol. **148**:68-71.

Garcia I, Lora JM, de la Cruz J, Benitez T, Llobell A and Pintor-Toro JA. 1994. Cloning and characterization of a chitinase (CHIT42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr. Genet. **27**:83-89.

Garrett SD. 1963. Soil fungi and soil fertility. Pergammon, London.

Garrett SD. 1970. Pathogenic Root-infecting Fungi. University Press, Cambridge.

Gaze RH. 1985. Cultivation systems and their evolution. In: Flegg PB, Spencer DM, Wood DA (eds) The biology and technology of the cultivated mushroom, John Wiley & Sons Ltd. pp.23-41.

Gea FJ, Pardo A, Navarro MJ and Pardo J. 1995. Mycoflora related to the mushroom spawn. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.557-562.

Geremia RA, Goldman GH, Jacobs D and Ardiles W, Vila SB, van Montagu M and Herrera-Estrella A. 1993. Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Molecular Microbiology **8**(3):603-613.

Gerrits JPG. 1969. Organic compost constituents and water utilised by the cultivated mushroom during spawn run and cropping. Mushroom Science **7**:111-126.

Gerrits JPG. 1977. The supplementation of horse manure compost and synthetic compost with chicken manure and other nitrogen sources. In: Hayes WA (ed), Composting: Improvements and future prospects. Proceedings of the second seminar in mushroom science, University of Aston. WS Maney & Son Ltd, Leeds, England.

Gerrits JPG. 1988. Nutrition and compost. In: van Griensven LJLD (ed) The cultivation of mushrooms. Darlington Mushroom Laboratories Ltd, Rustington, Sussex, England. pp.29-72.

Gerrits JPG, Amsing JGM, Straatsma G and van Griensven LJLD. 1995. Phase I process in tunnels for the production of *Agaricus bisporus* compost with special reference to the importance of

water. In: Elliot TJ (ed) Science and cultivation of edible fungi, volume 1, Balkema, Rotterdam. pp.203-211.

Ghisalberti EL and Sivasithamparam K. 1991. Review: Antifungal antibiotics produced by *Trichoderma* spp. Soil Biology and Biochemistry **23**(11):1011-1020.

Ghisalberti EL, Narbey MJ, Dewan MM and Sivasithamparam K. 1990. Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and produce pyrones. Plant and Soil **121**:287-291.

Ghose T. 1987. Measurement of cellulase activities. Commission on Biotechnology, IUPAC. Pure and Applied Chemistry **59**:258-268.

Goldman GH, Hayes C and Harman GE. 1994. Molecular and cellular biology of biocontrol by *Trichoderma* spp. TIBTECH **12**:478-482.

Gooday GW. 1994. Hormones in mycelial fungi. In: Wessels JGH, Meinhardt F (eds) The Mycota I: Growth, differentiation and sexuality, Springer-Verlag Berlin Heidelberg, pp 401-411.

Gooday GW. 1995. The dynamics of hyphal growth. Mycological Research **99** (4): 385-394.

Goyal A, Ghosh B and Eveleigh D. 1991. Characteristics of fungal cellulases. Bioresource Technology **36**:37-50.

Grime JP. 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. Am Nat **111**:1169-1194.

Grogan H and Fletcher JT. 1993. A new control for *Trichoderma* in mushroom compost. HDC Project News No. 21 September.

Grogan H and Young C. 1995. Controlling *Trichoderma* using carbendazim on spawn – further developments. HDC Project News No. 31 March.

Grogan H and Gaze RH. 1995. Growth of *Trichoderma harzianum* in traditional and experimental composts. In: Elliot (ed), Science and cultivation of edible fungi. pp653-660.

Haltrich D, Nidetzky B, Kulbe KD, Steiner W and Zupancic S. 1996. Production of fungal xylanases. Bioresource Technology **58**:137-161.

Handelsman J and Parke JL. 1989. Mechanism in biocontrol of soilborne plant pathogens. In: Kosuge T, Nester EW (eds) Plant microbe interactions, vol 3. McGraw-Hill, New York.

Hankin L and Anagnostakis SL. 1975. The use of solid media for detection of enzyme production by fungi. Mycologia **67**:597-607.

Haran S, Schickler H, Oppenheim A and Chet I. 1996. Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. Phytopathology **86**(9):980-985.

Haran S, Schickler H and Chet I. 1996a. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiol **142**:2321-2331.

Haran S, Schickler H, Oppenheim A and Chet I. 1995. New components of the chitinolytic system of *Trichoderma harzianum*. Mycological Research **99**(4):441-446.

Harmsen MC, Tolner B, Kram A, Go SJ, de Haan A and Wessels JGH. 1991. Sequences of three dsRNAs associated with La France disease of the cultivated mushroom (*Agaricus bisporus*). Curr Genet **20**:137-144.

Hayes CK, Klemsdal S, Lorito M, Di Pietro A, Peterbauer C, Nakas JP, Tronsmo A and Harman GE. 1994. Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum*. Gene **138**:143-148.

Hayes WA. 1977. Mushroom nutrition and the role of micro-organisms in composting. (ed). 1977. Composting: Improvements and future prospects. WS Maney and Son Ltd, Leeds, England.

Hayn M, Klinger R and Esterbauer H. 1993. Isolation and partial characterization of a low molecular weight endoglucanase from *Trichoderma reesei*. Proceedings of the second TRICEL symposium on *Trichoderma reesei* cellulases and other hydrolases, Espoo, Finland, ed. by Suominen P, Reinikainen T. Foundation for Biotechnical and Industrial Fermentation Research **8**:147-151.

Herrera-Estrella A, Goldman GH, Van Montagu M and Geremia RA. 1993. Electrophoretic karyotype and genes assignment to resolved chromosomes of *Trichoderma* spp. Mol Microbiol 7:515-521.

Hollings M. 1962. Viruses associated with a die back disease of the cultivated mushroom. Nature **196**:962-965.

Howell C. 1987. Relevance of mycoparasitism in the biological control of *Rhizoctonia solani* by *Gliocladium virens*. Phytopathology **77**:992-994.

Ilmen M, Onnela M-L, Klemsdal S, Keranen S and Penttila M. 1996. Functional analysis of the cellobiohydrolase I promoter of the filamentous fungus *Trichoderma reesei*. Mol Gen Genet **253**:303-214.

Ilmen M, Saloheimo A, Onnela M-L and Penttila ME. 1997. Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl Env Microbiol **63**(4):1298-1306.

Inbar J and Chet I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibres. J Bacteriol **174**:1055-1059.

Inbar J and Chet I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. Microbiology **141**:2823-2829.

Jeffries P. 1997. Mycoparasitism. In: D.T. Wicklow and B. Soderstrom (eds), The Mycota IV: Environmental and Microbial Relationships, Springer-Verlag, Berlin, Heidelberg.

Kalberer PP. 1984. Some properties of an extracellular proteolytic enzyme of *Verticillium fungicola*, a pathogen of the cultivated mushroom *Agaricus bisporus*. Phytopath. Z. **110**:213-220.

Kalberer PP. 1995. Factors influencing the dry matter content of the fruit bodies of *Agaricus bisporus*. In: Elliot TJ (ed) Science and cultivation of edible fungi, volume 1, Balkema, Rotterdam. pp.417-422.

Kamada T. 1994. Stipe elongation in fruit bodies. In: Wessels JGH, Meinhardt F (eds) The Mycota I: Growth, Differentiation and Sexuality. Springer-Verlag, Berlin, Heidelberg.

Kapat A, Zimand G and Elad Y. 1998. Effect of two isolates of *Trichoderma harzianum* on the activity of hydrolytic enzymes produced by *Botrytis cinerea*. Physiol and Molec Plant Pathol **52**:127-137.

Keddy PA. 1989. Competition. Chapman and Hall, London, New York.

Kent NL. 1989. Technology of cereals. Third edition. Pergamon Press, Oxford.

Kitamoto Y, Kono R, Tokimoto K, Mora N and Ichikawa Y. 1984. Production of lytic enzymes against cell walls of basidiomycetes from *Trichoderma harzianum*. Trans. Mycol. Soc. Japan. **26**:69-79.

Kleman-Leyer KM, Siika-Aho M, Teeri TT and Kirk TK. 1996. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. Appl Env Microbiol **62**(8):2883-2887.

Kubicek CP. 1993. From cellulose to cellulase inducers: facts and fiction. Proceedings of the second TRICEL symposium on *Trichoderma reesei* cellulases and other hydrolases, Espoo, Finland, ed. by Suominen P, Reinikainen T. Foundation for Biotechnical and Industrial Fermentation Research **8**:181-188.

Lehtineu U. 1993. Plant cell wall degrading enzymes of *Septoria nodorum*. Physiological and Molecular Plant Pathology **43**:121-134.

Lewis K, Whipps JM and Cooke RC. 1988. Mechanisms of biological disease control with special reference to the case study of *Pythium oligandrum* as an antagonist. In: Whipps JM, Lumsden RD (eds) Biotechnology of fungi for improving plant growth: Symposium of the British Mycological Society held at University of Sussex, September 1988.

Limon MC, Lora JM, Garcia I, de la Cruz J, Llobell A, Benitez T and Pintor-Toro JA. 1995. Primary structure and expression pattern of the 33 kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet **28**:478-483.

Linder M, Lindeberg G, Reinikainen T, Teeri TT and Petersson G. 1995. The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. FEBS Lett **372**:96-98.

Lora JM, de la Cruz J, Llobell A, Benitez T and Pintor-Toro JA. 1995. Molecular characterization and heterologous expression of an endo-β-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. **247**:639-645.

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL and Di Pietro A. 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology **83**:302-307.

Lorito M, Hayes CK, Di Pietro A, Woo SL and Harman GE. 1994. Purification, characterization, and synergistic acitivity of a glucan 1,3-β-glucosidase and an *N*-acetyl-β-glucosaminidase from *Trichoderma harzianum*. Phytopathology **84**:(4)398-405.

Lorito M, Farkas V, Rebuffat S, Bodo B and Kubicek CP. 1996a. Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J Bacteriology **178**: (21)6382-6385.

Lorito M, Mach RL, Sposato P, Strauss J, Peterbauer CK and Kubicek CP. 1996b. Mycoparasitic interaction relieves binding of the Cre 1 carbon catabolite repressor protein to promoter sequences of the *ech42* (endochitinase-encoding) gene in *Trichoderma harzianum*. Proc Natl Acad Sci USA **93**:14868-14872.

Lorito M, Woo SL, Garcia Fernandez I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S and Scala F. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA **95**:7860-7865.

Mach RL, Strauss J, Zeilinger S, Schindler M and Kubicek CP. 1996. Carbon catabolite repression of xylanase 1 (*xyl1*) gene expression in *Trichoderma reesei*. Molecular Microbiology **21**: (6)1273-1281.

MAFF. 1972. Mushroom Pests. Advisory leaflet 583.

Manchenko GP. 1994. Handbook of detection of enzymes on electrophoretic gels. CRC Press (Boca Raton, Ann Arbor, London, Tokyo.

Manocha MS. 1965. Fine structure of the *Agaricus* carpophore. Can J Bot **43**:1329-1333.

Manocha MS and Sahai AS. 1993. Mechanisms of recognition in necrotrophic and biotrophic mycoparasites. Can J Microbiol **39**:269-275.

Margolles-Clark E, Hayes CK and Harman GE and Penttila M. 1996. Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei.* Appl and Env Microbiology **62**: (6)2145-2151

Margolles-Clark E, Harman GE and Penttila M. 1996. Enhanced expression of endochitinase in *Trichoderma harzianum* with the *cbh1* promoter of *Trichoderma reesei.* Appl and Env Microbiology **62**: (6)2152-2155.

Masaphy S, Levanon D, Tchelet R and Henis Y. 1987. Scanning electron microscopy studies of interactions between *Agaricus bisporus* (Lang) Sing. hyphae and bacteria in casing. Appl Env Microbiol **53**:1132-1137.

Matcham SE and Wood DA. 1993. Purification of *Agaricus bisporus* extracellular laccase from mushroom compost. Biotechnology Letters **14**: (4)297-300.

McNeil M, Darvill AG, Fry SC and Albersheim P. 1984. Structure and function of the primary cell walls of plants. Ann Rev Biochem **53**:625-663.

Messner R and Kubicek CP. 1988. Intracellular precursors of endo-β-1,4-glucanase in *Trichoderma reesei.* FEMS Microbiology Letters **50**:227-232.

Michalenko GO, Hohl HR and Rast D. 1976. Chemistry and architecture of the mycelial wall of *Agaricus bisporus.* J Gen Microbiology **92**:251-262.

Mol PC and Wessels JGH. 1990. Differences in wall structure between substrate hyphae and hyphae of fruit-body stipes in *Agaricus bisporus.* Mycol Res **94**: (4)472-479.

Morris E, Doyle O and Clancy KJ. 1995a. A profile of *Trichoderma* species I – Mushroom compost production. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.611-618.

Morris E, Doyle O and Clancy KJ. 1995b. A profile of *Trichoderma* species II – Mushroom growing units. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.619-626.

Mumpuni A, Sharma HSS and Brown AE. 1998. Effect of metabolites produced by *Trichoderma harzianum* biotypes and *Agaricus bisporus* on their respective growth radii in culture. Appl Environ Microbiol **64**(12):5053-5056.

Muthumeenakshi S, Mills PR, Brown AE and Seaby DA. 1994. intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Microbiology **140**:769-777.

Muthumeenakshi S and Mills PR. 1995. Detection and differentiation of fungal pathogens of *Agaricus bisporus*. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.603-610.

Muthumeenakshi S, Brown AE and Mills PR. 1998. Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. Mycol Res **102** (4):385-390.

Nelson NJ. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153**:375-380.

Noronha EF and Ulhoa CJ. 1996. Purification and characterization of an endo-β-1,3-glucanase from *Trichoderma harzianum*. Can J Microbiol **42**:1039-1044.

Novaes-Ledieu M and Garcia Mendoza C. 1981. The cell walls of *Agaricus bisporus* and *Agaricus campestris* fruiting body hyphae. Can J Microbiology **27**:779-787.

O'Connell RJ, Bailey JA and Richmond DV. 1985. Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Physiol. Plant Pathol. **27**:75-98.

Ohmasa M, Tsunoda M and Hiraide M. 1995. A method to assay varietal differences of disease resistance of *Lentinus edodes* against *Trichoderma* species. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.579-585.

Ospina-Giraldo MD, Royse DJ, Chen X and Romaine P. 1997a. Application of molecular technologies for the study of mushroom green mold-associated strains of *Trichoderma*. Mushroom News **45**: (9)34-37.

Ospina-Giraldo MD, Thon MR, Chen X, Romaine CP and Royse DJ. 1997b. Phylogeny of *Trichoderma* spp. associated with mushroom green mold in Europe and North America. Phytopathology **87**:S73 Publication No. P-1997-0519-AMA.

Ospina-Giraldo MD, Watson C, Gueldner R, Chen X, Romaine CP and Royse DJ. 1997c. *In vitro* evaluation of biological fungicides for control of Trichoderma green mold of the button mushroom, *Agaricus bisporus*. Phytopathology **87**:S73. Publication no. P-1997-0520-AMA.

Ospina-Giraldo MD, Royse DJ, Thon MR, Chen X and Romaine CP. 1998. Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia **90**: (1)76-81.

Papavizas GC. 1985. Trichoderma and Gliocladium: Biology, ecology and potential for biocontrol. Ann Rev Phytopathol **23**:23-54.

Papavizas GC and Lumsden RD. 1982. Improved medium for isolation of *Trichoderma* spp. from soil. Plant Disease **66**:1019-1020.

Papavizas GC and Lewis JA. 1989. Effect of *Gliocladium* and *Trichoderma* on damping-off and blight of snap bean caused by *Sclerotium rolfsii* in the greenhouse. Plant Pathol **38**:277-286.

Paterson IC, Charnley AK, Cooper RM and Clarkson JM. 1993. Regulation of production of a trypsin-like protease by the insect pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiology Letters **109**:323-328.

Paterson IC, Charnley AK, Cooper RM and Clarkson JM. 1994. Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiology **140**:185-189.

Paterson RRM and Bridge PD. 1994. Enzymatic activity on solid media. In: IMI Technical Handbooks No.1. Biochemical Techniques for Filamentous Fungi. CAB International.

Peberdy JF. 1990. Fungal Cell Walls – A Review. In: Biochemistry of cell walls and membranes in fungi. Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW and Copping LG (eds). Springer-Verlag, London.

Pelczar MJ, Chan ECS and Krieg NR. 1986. Microbiology. Fifth edition. McGraw-Hill Book Company, London.

Penttila M, Lehtovaara P, Nevalainen H, Bhikhabhai R and Knowles J. 1986. Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. Gene **45**:253-263.

Pianka ER. 1970. On r and K selection. Am Nat **102**:592-597.

Pizer NH and Thompson AJ. 1938. Investigations into the environment and nutrition of the cultivated mushroom. Part 2. The effect of calcium and phosphate on growth and productivity. J Agric Sci **28**:604-617.

Poppe JA and Hofte M. 1995. Twenty wastes for twenty cultivated mushrooms. In: Elliot TJ (ed) Science and cultivation of edible fungi, volume 1, Balkema, Rotterdam. pp.171-180.

Price PW. 1984. Alternative paradigms in community ecology. In: A New Ecology: Novel Approaches to Interactive Systems (eds PW Price, CN Slobidchikoff and WSA Gaud) Wiley, New York.

Puyesky M, Ponce-Noyola P, Horwitz BA and Herrera-Estrella A. 1997. Glyeraldehyde-3-phosphate dehydrogenase expression in *Trichoderma harzianum* is repressed during conidiation and mycoparasitism. Microbiology **143**:3157-3164.

Raeda U and Broda P. 1985. Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology **1**(1):17-20.

Raguz S, Yague E, Wood DA and Thurston CF. 1992. Isolation and characterisation of a cellulose-growth-specific gene from *Agaricus bisporus*. Gene **119**:183-190.

Rainey PB. 1989. Short Communication: A new laboratory medium for the cultivation of *Agaricus bisporus*. New Zealand Natural Sciences **16**:109-112.

Randle PE. 1977. Aeration requirement in composting. In: Hayes WA (Ed), Composting: Improvements and future prospects. Proceedings of the second seminar in mushroom science, University of Aston. WS Maney & Son Ltd, Leeds, England.

Ratto M, Niku-Paavola ML, Raaska L, Mattila-Sandholm T and Viikari L. 1996. The effect of *Trichoderma harzianum* siderophores on yeasts and wood-rotting fungi. Material und Organismen **30**: (4)279-292.

Rayner ADM. 1986. Mycelial interactions – genetic aspects. Natural Antimicrobial Systems FEMS Symposium 35, Bath University Press.

Ridout CJ, Coley-Smith JR and Lynch JM. 1988. Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Micro Technol **10**:180-187.

Rifai MA. 1969. A revision of the genus *Trichoderma*. Mycological Papers No. 116:1-55.

Rinker DL and Alm G. 1997a. Effect of supplementation at spawning or casing on the expression of *Trichoderma harzianum*, biotype Th4, in commercial mushroom production. Mushroom News **45**: (11)6-11.

Rinker DL and Alm G. 1997b. Investigations of factors influencing the expression of green mold. Mushroom World June 1997: 25-29.

Rinker DL and Alm G. 1997c. Not all green is *Trichoderma* green mold. Mushroom World Sept 1997: 47-50.

Rinker DL, Alm G, Castle A and Rghei N. 1997. Distribution of green mold on infected mushroom farms. Mushroom World June 1997: 71-75.

Rinker DL. 1996. Trichoderma disease: progress toward solutions. Mushroom World. December: 46-54.

Romaine CP, Wuest PJ, Royse DJ and Beyer DM. 1997. Mushroom green mold: cause, edaphic factors and control. Mushroom News **45**: (10)28-33.

Romaine CP, Royse DJ, Wuest PJ and Beyer DM. 1996. Mushroom green mold: cause, edaphic factors and control. Mushroom News. November: 20-23.

Royse DJ, Ospina-Giraldo M, Thon MR, Chen X and Romaine CP. 1998. Potential threat to *Pleurotus* spp. production from *Trichoderma harzianum* (Th4), cause of mushroom green mold of *Agaricus bisporus*. Mushroom News **46**: (2)20-24.

Saloheimo M, Lehtovaara P, Penttila M, Teeri TT, Stahlberg J, Johansson G, Petterson G, Claeyssens M, Tomme P and Knowles JKC. 1988. EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. Gene **63**:11-21.

Salvesen G and Nagase H. 1989. Inhibition of proteolytic enzymes. In: Beynon RJ, Bond JS (eds) Practical Approach: Proteolytic Enzymes, IRL Press.

Samuels GJ. 1996. *Trichoderma*: a review of biology and systematics of the genus. Mycol Res **100**: (8)923-935.

Scalbert A, Monties B, Lallemand J-Y, Guittet E and Rolando C. 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry **24**(6):1359-1362.

Schickler H, Danin-Gehali BC, Haran S and Chet I. 1998. Electrophoretic characterization of chitinases as a tool for the identification of *Trichoderma harzianum* strains. Mycol Res **102**(3):373-377.

Schirmbock M, Lorito M, Wang Y-L, Hayes CK, Arisan-Atac I, Scala F, Harman GE and Kubicek CP. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl Env Microbiol **60**(12):4364-4370.

Schisler LC and Patton TG. 1970. Stimulation of yield in the cultivated mushroom by vegetable oils – effects of sterols and ethyl linoleate. J Agr Food Chem **18**(6):1102-1103.

Schmuck M and Pilz I. 1986. Investigation of cellobiohydrolase from *Trichoderma reesei* by small angle x-ray scattering. Biotechnology Letts **8** (6):397-402.

Schoeman MW, Webber JF and Dickinson DJ. 1996. The effect of diffusible metabolites of *Trichoderma harzianum* on *in vitro* interactions between basidiomycete isolates at two different temperature regimes. Mycol Res **100**: (12)1454-1458.

Schulein M, Tikhomirov DF and Schou C. 1993. *Humicola insolens* alkaline cellulases. Proceedings of the second TRICEL symposium on *Trichoderma reesei* cellulases and other hydrolases, Espoo, Finland, ed. by Suominen P, Reinikainen T. Foundation for Biotechnical and Industrial Fermentation Research **8**:109-116.

Schwarz WH, Bronnenmeier K, Grabwitz F and Staudenbauer WL. 1987. Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans. Analytical Biochemistry **164**:72-77.

Seaby DA. 1987. Infection of mushroom compost by *Trichoderma harzianum.* Mushroom J **179**:355-361.

Seaby DA. 1989. Further observations on *Trichoderma* species. Mushroom J **197**: 147-151.

Seaby DA. 1996a. Differentiation of *Trichoderma* taxa associated with mushroom production. Plant Pathology **45**:905-912.

Seaby DA. 1996b. Investigation of the epidemiology of green mould of mushroom (*Agaricus bisporus*) compost caused by *Trichoderma harzianum.* Plant Pathology **45**:913-923.

Seiboth B, Hakola S, Mach RL, Suominen PL and Kubicek CP. 1997. Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei.* J Bacteriol **179**(17):5318-5320.

Sharma A and Nakas JP. 1987. Preliminary characterization of laminarinase from *Trichoderma longibrachiatum.* Enzyme Microb Technol **9**:89-93.

Sharma HSS, Kilpatrick M, Ward F, Lyons G and Burns L. 1999. Colonisation of phase II compost by biotypes of *Trichoderma harzianum* and their effect on mushroom yield and quality. Appl Microbiol Biotechnol **51**:572-578.

Sharon N and Lis H. 1989. Lectins. Chapman and Hall, London.

Shearer CA. 1995. Fungal Competition. Can J Bot **73**(Suppl 1):S1259-S1264.

Shoemaker S, Schweichart V, Ladner M, Gelfand D, Kwok S, Myambo K and Innis M. 1983. Molecular cloning of exo-cellobiohydrolase I derived from *Trichoderma reesei* strain L27. BIO/TECH **1**:691-696.

Sietsma JH and Wessels JGH. 1994. Apical wall biogenesis. In: Wessels JGH, Meinhardt F (eds) The Mycota I: Growth, differentiation and sexuality, Springer-Verlag Berlin Heidelberg.

Sinden JW and Hauser E. 1950. Report on two new mushroom diseases. Mushroom Sci **1**:96-100.

Sinden JW and Hauser E. 1953. Nature and control of three mildew diseases of mushrooms in America. Mushroom Sci **2**:177-180.

Sinsabaugh RL and Liptak MA. 1997. Enzymatic conversion of plant biomass. In: Wicklow DT, Soderstrom B (eds) The Mycota IV: Environmental and microbial relationships, Springer-Verlag, Berlin, Heidelberg.

Sivan A, Elad Y and Chet I. 1984. Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. Phytopathol **74**(4):498-501.

Sivan A and Chet I. 1986. Biological control of *Fusarium* spp. in cotton, wheat and muskmelon by *Trichoderma harzianum*. Phytopathol Z **116**:39-47.

Sivan A, Ucko O and Chet I. 1987. Biological control of Fusarium crown rot of tomato by *Trichoderma harzianum* under field conditions. Plant Disease **71**:587-592.

Sivan A and Chet I. 1989. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J Gen Microbiol **135**:675-682.

Somogyi M. 1952. Notes on sugar determination. J Biol Chem **195**:19-23.

Southwood TRE. 1977. Habitat, the template for ecological strategies? J Animal Ecology **46**:337-365.

Spiro RG. 1960. Studies on fetuin, a glycoprotein of fetal serum. J Biol Chem **235**(10):2860-2869.

Srisodsuk M, Lehtio J, Linder M, Margolles-Clark E, Reinikainen T and Teeri TT. 1997. *Trichoderma reesei* cellobiohydrolase I with an endoglucanase cellulose-binding domain: action on bacterial microcrystalline cellulose. J Biotech **57**:49-57.

St. Leger RJ, Charnley AK and Cooper RM. 1986. Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J Invertebrate Pathology **48**:85-95.

St. Leger RJ, Charnley AK and Cooper RM. 1987a. Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem and Biophys **253**: (1)221-232.

St. Leger R, Cooper RM and Charnley AK. 1987b. Production of cuticle-degrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. J Gen Microbiology **133**: 1371-1382.

St. Leger RJ, Cooper RM and Charnley AK. 1991. Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. J Invertebrate Pathology **58**:415-426.

Stanek M. 1972. Microorganisms inhabiting mushroom compost during fermentation. Mushroom Sci **8**:797-811.

Staunton L. 1986/1987. Mushroom Newsletters No. 6 and No. 7. Kinsealy Research Centre.

Staunton L. 1987. *Trichoderma* green mould in mushroom compost. Mushroom J **179**:362-363.

Straatsma G, Samson RA, Olijnsma TW, Op den Camp HJM, Gerrits JPG and van Griensven LJLD. 1994. Ecology of thermophilic fungi in mushroom compost, with emphasis on *Scytalidium thermophilum* and growth stimulation of *Agaricus bisporus* mycelium. Appl Env Microbiol **60**(2):454-458.

Straatsma G, Olijsma TW, van Griensvan LJDL and Op den Camp HJM. 1995. Growth promotion of *Agaricus bisporus* mycelium by *Scytalidium thermophilum* and CO_2. In: Elliot TJ (ed) Science and cultivation of edible fungi, volume 1, Balkema, Rotterdam. pp.289-291.

Stoltzer S and Grabbe K. 1991. Mechanisms of substrate selectivity in the cultivation of edible fungi. In: Maher (ed), Science and cultivation of edible fungi, Balkema, Rotterdam.

Tangerone B, Royer JC and Nakas JP. 1989. Purification and characterization of an endo-(1,3)-β-D-glucanase. Appl and Env Microbiol **55**: (1)177-184.

Teeri TT, Salovuori I and Knowles J. 1983. The molecular cloning of the major cellulases gene from *Trichoderma reesei*. BIO/TECH **1**:696-699.

Teeri TT, Lehtovaara P, Kauppinens S, Salovuori I and Knowles J. 1987. Homologous domains in *Trichoderma reesei* cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II. Gene **51**:43-52.

Teeri TT, Jones A, Kraulis P, Rouvinen J, Penttila M, Harkki A, Nevelainen H, Vanhanen S, Saloheimo M and Knowles JKC. 1990. Engineering *Trichoderma* and its cellulases. In: Kubicek CP, Eveleigh DE, Esterbauer H, Steiner W, Kubicek-Pranz EM (eds) *Trichoderma reesei* cellulases: biochemistry, genetics, physiology and applications. Proc TRICEL 89 Meet. held in Vienna, Sept 1989. Royal Society of Chemistry.

Teeri TT, Koivula A, Linder M, Wohlfahrt G, Divne C and Jones TA. 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? Biochem Soc Trans **26**:173-178.

Tenkanen M, Puls J and Poutanen K. 1992. Two major xylanases of *Trichoderma reesei*. Enzyme Microbiol Technol **14**:566-574.

Terras MA and Hales DF. 1995. Red pepper mites are vectors of *Trichoderma*. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.485-490.

Terwisscha van Scheltinga AC, Armand S, Kalk KH, Isogai A, Henrissat B and Dijkstra BW. 1995. Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and x-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. Biochem **34**:15619-15623.

Thompson DP and Eribo BE. 1984. Extracellular enzyme production by *Rhizopus* and *Mucor* species on solid media. Can J Microbiol **30**:126-128.

Thornton CR, Dewey FM and Gilligan CA. 1994. Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the detection of live propagules of *Trichoderma harzianum* in a peat-bran medium. Soil Biol Biochem **26**: (7)909-920.

Thornton CR and Gilligan CA. 1999. Quantification of the effect of the hyperparasite *Trichoderma harzianum* on the saprotrophic growth dynamics of *Rhizoctonia solani* in compost using a monoclonal antibody-based ELISA. Mycol Res **103**(4):443-448.

Thrane C, Tronsmo A and Jensen DF. 1997. Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. European J Plant Pathology **103**:331-344.

Tilman D. 1982. Resource Competition and Community Structure. Princeton University Press, Princeton, New Jersey.

Todorovic R, Grujic and Petrovic J. 1990. Some properties of)-β-D-glucosidase from *Trichoderma harzianum* C_1R_1. British Polymer J **22**:221-225.

Tokimoto K and Komatsu M. 1975. Effect of carbon and nitrogen sources in media on the hyphal interference between *Lentinus edodes* and some species of *Trichoderma*. Ann Phytopath Soc Japan **45**:261-264.

Tokimoto K, Fujita T, Takeda Y and Takaishi Y. 1987. Increased or induced formation of antifungal substrates in cultures of *Lentinus edodes* by the attack of *Trichoderma* spp. Proc Japan Acad **63**, Ser. B:277-280.

Tokimoto K, Komatsu M and Fukumasa-Nakai Y. 1994. Establishing the selection method for the *Lentinula edodes* strains resistant to *Trichoderma* spp. Proc Japan Acad **70**: ser. B:112-116.

Tokimoto K and Komatsu M. 1995. Selection and breeding of shiitake strains resistant to *Trichoderma* spp. Can J Bot **73** (suppl 1):S962-966.

Tokimoto K, Fukumasa-Nakai Y, Matsumoto T and Maekawa N. 1998. Selection of shiitake strains greatly competitive to antagonistic fungi *Trichoderma* spp. Reports of the Tottori Mycological Institute No. 36. S23.

Tomme P, Heriban V and Claeyssens M. 1990. Adsorption of two cellobiohydrolases from *Trichoderma reesei* to Avicel: evidence for "exo-exo" synergism and possible "loose complex" formation. Biotech Letts **12**(7):525-530.

Torronen A, Mach RL, Messner R, Gonzalez R, Kalkkinen N, Harkki A and Kubicek CP. 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnology **10**:1461-1465.

Torronen A, Harkki A and Rouvinen J. 1994. Three-dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site. The EMBO J **13**(11):2493-2501.

Torronen A and Rouvinen J. 1995. Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. Biochemistry **34**:847-856.

Townsend BB. 1954. Morphology and development of fungal rhizomorphs. Trans Brit Mycol Soc **37**:22-233.

Treschow C. 1944. Nutrition of the cultivated mushroom. Dansk Botanisk Arkiv **11**:(6)-(9).

Tressl R, Bahri D and Engel K-H. 1982. Formation of eight carbon and ten carbon components in mushrooms (*Agaricus campestris*). J Agric Food Chem **30**:89-93.

Tronsmo A and Harman GE. 1993. Detection and quantification of *N*-acetyl-*β*-D-glucosaminidase, chitobiosidase and endochitinase in solutions and on gels. Analytical Biochemistry **208**:74-79.

Tunney J. 1977. Practical considerations in the preparation of synthetic compost – compost without horse manure. In: Hayes WA (ed), Composting: Improvements and future prospects. Proceedings of the second seminar in mushroom science, University of Aston. WS Maney & Son Ltd, Leeds, England.

Turner EM, Wright M, Ward T, Osborne DJ and Self R. 1975. Production of ethylene and other volatiles and changes in cellulase and laccase activities during the life cycle of the cultivated mushroom *Agaricus bisporus*. J Gen Microbiol **91**:167-176.

Turner EM. 1977. Enzyme activity of *Agaricus bisporus* in compost. In: Hayes WA (ed), Composting: Improvements and future prospects. Proceedings of the second seminar in mushroom science, University of Aston. WS Maney & Son Ltd, Leeds, England.

Ulhoa CJ and Peberdy JF. 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol **137**:2163-2169.

Van Arsdell JN, Kwok S, Schweickart VL, Ladner MB, Gelfand DH and Innis MA. 1987. Cloning, characterization and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei*. BIO/TECH **5**:60-64.

Van Den Boogert PHJF and Deacon JW. 1994. Biotrophic mycoparasitism by *Verticillium biguttatum* on *Rhizoctonia solani*. European J. Plant Pathology **100**: 137-156.

Van der Lende TR, Harmsen MC and Wessels JGH. 1994. Double-stranded RNAs and proteins associated with the 34nm virus particle of the cultivated mushroom *Agaricus bisporus*. J Gen Virol **75**:2533-2536.

Van Gils JJ. 1988. Cultivation. In: van Griensven LJLD (ed) The cultivation of mushrooms. Darlington Mushroom Laboratories Ltd, Rustington, Sussex, England. pp.263-308.

Van Tilbeurgh H, Tomme P, Claeyssens M, Bikhabhai R and Pettersson G. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*: separation of functional domains. FEBS Lett **204**:223-237.

Van Zaayen A and Gams W. 1982. Contribution to the taxonomy and pathogenicity of fungicolous *Verticillium* species. II. Pathogenicity. Neth J Pl Path **88**:143-154.

Vasseur V, van Montagu M and Goldman GH. 1995. *Trichoderma harzianum* genes induced during growth on *Rhizoctonia solani* cell walls. Microbiology **141**:767-774.

Vazquez-Garciduenas S, Leal-Morales CA and Herrera-Estrella A. 1998. Analysis of the β-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. Appl Env Microbiol **64**(4):1442-1446.

Visscher HR. 1988. Casing Soil. In: van Griensven LJLD (ed) The cultivation of mushrooms. Darlington Mushroom Laboratories Ltd, Rustington, Sussex, England. pp.73-89.

Wach MP, Sriskantha A and Romaine CP. 1987. Double-stranded RNAs associated with La France disease of the commercial mushroom. Phytopathology **77**(9):1321-1325.

Waksman G. 1991. Purification and characterization of two endo-β-1,4-D-glucanases from *Sclerotinia sclerotiorum*. Biochimica et Biophysica Acta **1073**:49-55.

Wang MC and Bartnicki-Garcia S. 1970. Structure and composition of walls of the yeast form of *Verticillium albo-atrum*. J Gen Microbiol **64**:41-54.

Wannet WJB, Op den Camp HJM, van der Drift C and van Griensven LJLD. 1995. Aspects of the carbon metabolism of *Agaricus bisporus*. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.781-786.

Wardle DA, Parkinson D and Waller JE. 1993. Interspecific competitive interactions between pairs of fungal species in natural substrates. Oecologia **94**:165-172.

Weber RWS and Pitt D. 1997. Purification, characterization and exit routes of two acid phosphatases secreted by *Botrytis cinerea*. Mycol Res **101**:1431-1439.

Wells HD, Bell DK and Jaworski CA. 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. Phytopathol **62**:442-447.

Wessels JGH. 1993. Wall growth, protein excretion and morphogenesis in fungi. New Phytol **123**:397-413.

Wessels JGH. 1996. Fungal hydrophobins: proteins that function at an interface. Trends in Plant Sciences **1**: (1)9-15.

Whetten R and Sederoff R. 1995. Lignin Biosynthesis. The Plant Cell 7:1001-1013.

Whipps JM. 1987a. Effect of media on growth and interactions between a range of soilborne glasshouse pathogens and antagonistic fungi. New Phytol **107**:127-142.

Whipps JM, Lewis K and Cooke RC. 1987b. Mycoparasitism and plant disease control. In: Burge MN (Ed) Fungi in biological control systems, Manchester University Press.

Whipps JM and Magan N. 1987c. Effects of nutrient status and water potential of media on fungal growth and antagonist-pathogen interactions. Bulletin OEPP/EPPO Bulletin **17**:581-591.

White PF. 1985. Pests and Pesticides. In: Flegg PB, Spencer DM, Wood DA (Eds) The biology and technology of the cultivated mushroom, John Wiley & Sons Ltd, pp.279-293.

White PF. 1995. Biological control of mushroom pests: An evaluation. In: Elliot (ed) Science and cultivation of edible fungi, Balkema, Rotterdam. pp.475-484.

Widden P. 1997. Competition and the fungal community. In: Wicklow DT, Söderström (eds) The Mycota IV: Environmental and Microbial Relationships, Springer-Verlag Berlin Heidelberg, pp 135-147.

Widden P and Abitbol JJ. 1980. Seasonality of *Trichoderma* species in a spruce forest soil. Mycologia **72**:775-784.

Widden P and Hsu D. 1987. Competition between *Trichoderma* species: effects of temperature and litter type. Soil Biol Biochem **19**(1):89-93.

Widden P and Scattolin V. 1988. Competitive interactions and ecological strategies of *Trichoderma* species colonising spruce litter. Mycologia **80**(6):795-803.

Woo SL, Donzelli B, Scala F, Mach R, Harman GE, Kubicek CP, Del Sorbo G and Lorito M. 1999. Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Molec Plant-Microbe Interactions **12**(5):419-429.

Wood DA. 1976. Primodium formation in axenic cultures of *Agaricus bisporus* (Lange) Sing. J Gen Microbiol **95**:313-323.

Wood DA. 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. J Gen Microbiol **117**:327-338.

Wood DA, Craig GD, Atkey PT, Newsam RJ and Gull K. 1985. Ultrastructural studies on the cultivation processes and growth and development of the cultivated mushroom *Agaricus bisporus*. Food Microstructure **4**:143-164.

Wood DA and Fermor TR. 1985. Nutrition of *Agaricus bisporus*. In: Flegg PB, Spencer DM, wood DA (eds) The biology and technology of the cultivated mushroom, John Wiley & Sons Ltd. pp.43-61.

Wood DA and Goodenough PW. 1977. Fruiting of *Agaricus bisporus*. Arch Microbiol **114**:161-165.

Wood DA and Hammond JBW. 1977. Inhibition of growth and development of *Agaricus bisporus* by Polyoxin D. J Gen Microbiol **98**:625-628.

Wood TM and Mahalingeshwara Bhat K. 1988. Methods for measuring cellulase activities. Methods in Enzymology **160**:87-112.

Wood TM and Garcia-Campayo V. 1990. Enzymology of cellulose degradation. Biodegradation **1**:147-161.

Wosten HAB, de Vries OMH, and Wessels JGH. 1993. Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. The Plant Cell **5**:1567-1574.

Wuest PJ, Duffy MD and Royse DJ. 1981. Six steps to mushroom farming (special circular 268). The Pennsylvania State University, College of Agriculture, Extension Service, University Park, Pennsylvania.

Yagüe E, Wood DA and Thurston CF. 1994. Regulation of transcription of the *cel1* gene in *Agaricus bisporus*. Molecular Microbiology **12**: (1)41-47.

Yodzis P. 1986. Competition, mortality and community structure. In: Community Ecology (eds J Diamond and TJ Case) Harper and Row, New York.

Zeilinger S, Mach RL, Schindler M, Herzog P and Kubicek CP. 1996. Different inducibility of expression of the two xylanase genes *xyn1* and *xyn2* in *Trichoderma reesei*. J Biol Chem **271**(41):25624-25629.

Zhao J, de Koker TH and Janse BJH. 1996. Comparative studies of lignin peroxidases and Mn^{+}-dependent peroxidases produced by selected white rot fungi in solid media. FEMS Microbiol Lett **145**:393-399.

Appendix A

Media, substrates and reagents

A.1. Agar media

The agar media used in plate assays (*in vitro*) contained the following ingredients (l^{-1}):

Malt extract agar (MEA)

20 g malt extract
20 g tissue culture agar
1 L distilled water
Autoclaved for 15 min at 15 lb. per sq. inch

Distilled water agar (DWA)

20 g tissue culture agar
1 L distilled water
Autoclaved for 15 min at 15 lb. per sq. inch

Filtered compost agar (fCA)
(modified from Rainey 1989)

50 g blended oven-dried compost
20 g tissue culture agar
1 L compost filtrate
The blended compost was infused in 1L of distilled water for three hours and was subsequently filtered through four layers of muslin. Clarified filtrate was made up to 1L and agar was added prior to sterilisation in an autoclave (15 min at 15 lb. per sq. inch).

Blended compost agar (bCA)

1 g blended autoclaved compost per petri dish

20 ml DWA per dish

A.2. Preparation of rye grain as an inoculum base for T. harzianum conidia.

Glass jars were filled with rye grain and autoclaved for 15 min at 15 lb. per sq. inch and then cooled to room temperature. Sterile grain was then stored at 4°C.

A.3. Preparation of colloidal chitin.

100 g of fine powdered technical grade chitin (Sigma, UK) was dissolved in 1L of concentrated (85%) H_3PO_4 for 48 hours at 4°C. This was then filtered through glass wool and poured into vigorously stirred 50% (v/v) ethanol to precipitate the chitin. The residue was left to sediment and was then resuspended in distilled water several times, to remove excess acid and ethanol and subsequently dialysed against running tap water for 48 hours to remove chitin oligosaccharides and other low molecular weight impurities. The colloidal suspension was then resuspended in sterile 50 mM citrate

buffer [pH 6.0] and stored at 4°C. Sodium azide (0.02% w/v) was added to prevent contamination.

A.4. Nelson Reagents for sugar determination.

Reagent A

25 g Na_2CO_3
25 g sodium potassium tartrate
20 g $NaHCO_3$
200 g Na_2SO_4
800 ml distilled water (dissolve ingredients make up to 1L and filter)

Reagent B
15% (w/v) $CuSO_4.5H_2O$
1 or 2 drops of concentrated H_2SO_4 per 100 ml
Reagents A and B were mixed in the ratio 25:1 and allowed to stand for 30 min before use.

Reagent C
(Made in clean, acid washed glassware)
25 g ammonium molybdate
450 ml distilled water - when dissolved add:
21 ml concentrated H_2SO_4
Separately dissolve:
3 g $Na_2HAsO_4.7H_2O$ in 25 ml of distilled water and add to ammonium molybdate solution.
Store in a brown bottle at room temperature.

A.5. Reagents for fixing and staining IEF gels.

Fixing solution

29 g TCA
8.5 g sulphosalicyclic acid
Dissolved in distilled water and made up to 250 ml. Stored for 3 months at 20 to 30°C.

Destaining or washing solution

500 ml ethanol
160 ml acetic acid
Made up to 2L with distilled water and stirred. Stored for 2 weeks at 20 to 30°C.

Staining solution

0.5% (w/v) Coomassie blue
50% (v/v) ethanol
7.5% (v/v) acetic acid
Stored for 2 months at 20 to 30°C.

Preserving solution

25 ml glycerol
Made up to 250 ml by adding destaining solution. Stored for 2 weeks at 20 to 30°C.

Staining and preserving IEF gels

(according to Ampholine® PAGplate instructions, Pharmacia Biotech, UK)

Step No.	Solution	Incubation time (min)
1	Fixing solution	30 to 60
2	Washing solution	5
3	Staining solution	120 to 180
4	Destaining solution	until background is clear
5	Preserving solution	60

A.6. Solid media for the detection of enzyme activity.

Particulate or dyed substrates were incorporated into solid media to detect the activity of extracellular enzymes. Activity was observed as a clearing in the dye or particulate substance adjacent to the advancing edge of the fungal colony. The following were used for the detection of extracellular enzyme activity of *T. harzianum*.

Chitinase medium

Underlayer: 1.5% agar in distilled water was sterilised (121°C for 15 min), poured and allowed to set. The overlayer comprised a mineral salts solution: 0.08g $(NH_4)_2SO_4$, 0.16g KH_2PO_4, 0.24g Na_2HPO_4, 0.4ml Cooper and Wood (1975) trace element stock (see Chapter Two 2.2.1) and made up to 40ml with distilled water, this was then added to the test medium: 0.02% yeast extract, 1.5% agar and 2.4% purified chitin. When the underlayer was sufficiently set, 3ml overlayer was added and allowed to set.

Isolate	C*	Std. Error of C	C + Z+	Std. Error of C+Z	C+Z/C
T7	28.33	0.498	32.42	0.917	1.144
Th2A	32.5	0.544	35.58	0.657	1.095
Th1(c)	33.00	2.449	35.00	2.082	1.06

* denotes colony diameter (mm); + denotes diameter of colony and clearing zone (mm). Strains T7 and Th2A (Th2) and Th1(c) (Th1).

Fatty acid esterase medium

(l^{-1}): 10g mycological peptone, 5g NaCl, 0.1g $CaCl_2.2H_2O$, 15g agar and 25mg bromocresol purple, pH to 5.4 using 1M NaOH (autoclaved at 121°C for 10 min). A

10% solution of Tween 20 was autoclaved separately and added to the medium to give a final concentration of 1%.

Isolate	C	Std. error of C	C+Z	Std. error of C+Z	C+Z/C
T7	32.75	0.351	37.67	0.4495	1.15
T32	40.14	0.404	47.0	0.348	1.17
Th1(c)	35.64	0.498	43.0	0.363	1.21
TD15	37.71	0.398	41.21	0.261	1.09

See table above for explanatory notes. Th2: T7 and Th2A; Th1: Th1(c) and TD15.

Xylanase medium

(Modification, Farkas *et al*, 1985)

2% agar and 1% oxgall (Sigma) was added to a basal medium (Cooper and Wood 1975) with carbon source omitted. Remazol brilliant blue-xylan (Sigma, UK) was autoclaved separately, dissolved in 1-2 ml sterile distilled water and added at a final concentration of 0.2% (v/v) to the medium before dispensing.

Isolate	C	Std. error of C	C+Z	Std. error of C+Z	C+Z/C
T7	31.67	0.333	40.0	0.447	1.263
Th1(c)	43.17	0.477	48.0	0.258	1.112

See table above for explanatory notes.

Lignase medium

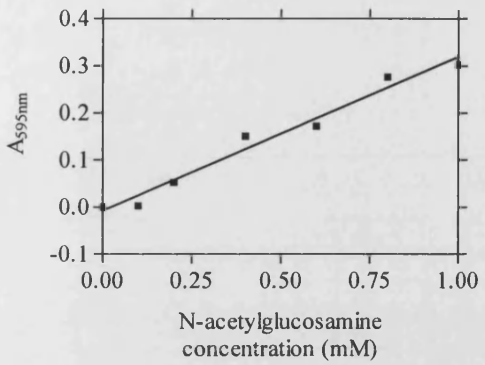
(l^{-1}): 0.6g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.4g K_2HPO_4, 0.22g $(NH_4)_2$ tartrate, 40g sorbose, 0.2g PolyR-478 dye (Sigma), 15g agar made up in distilled water. Stock mineral solution (as for lectin study see Appendix B) was added at a rate of 1 ml per 1 L of medium. The medium was autoclaved (121°C for 15 minutes) and after cooling, 100µl L^{-1} thiamine hydrochloride (filter sterilised) was added from a of 10mg ml^{-1} stock. *T. harzianum* displayed no lignase activity on this medium.

A.7. Calibration curves for quantification of depolymerase activity.

Calibration curves of a known range of concentrations of relevant monomers were produced to enable quantification of the enzyme activity produced by *T. harzianum*. A concentration range of 0, 0.2, 0.4, 0.6 and 0.8 mM was used for N-acetylglucosamine, glucose and xylose, while p-nitroaniline was used at concentrations of 15.63, 31.25,

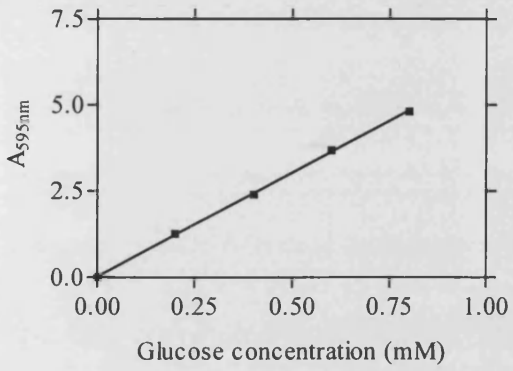
62.5 and 125 μM. Absorbency readings were plotted versus sugar concentrations and the linear regression calculated. The calculation of activity as nkat/ml was performed as described in the Methodology (section 2.3.0).

a



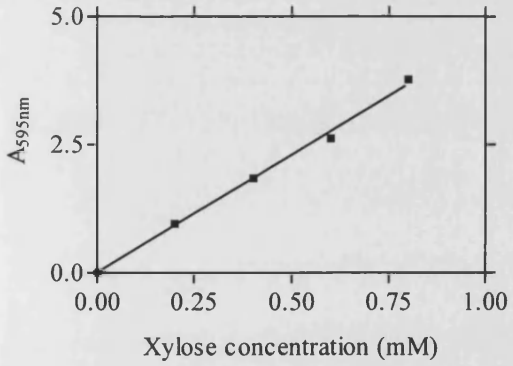
Variables	
Slope	0.3267 ± 0.02396
Y-intercept	-0.007962 ± 0.01346
X-intercept	0.02437
1/slope	3.061
95% Confidence Intervals	
Slope	0.2651 to 0.3883
Y-intercept	-0.04257 to 0.02665
Goodness of Fit	
r^2	0.9738

b



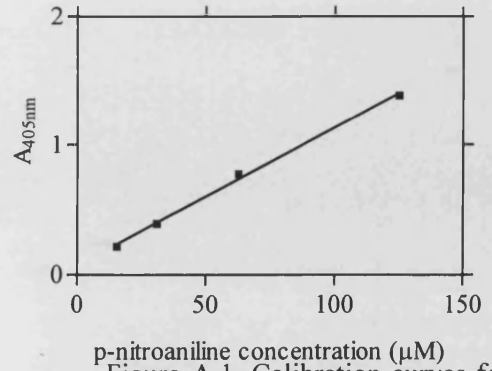
Variables	
Slope	6.013 ± 0.07211
Y-intercept	0.02600 ± 0.03533
X-intercept	-0.004324
1/slope	0.1663
95% Confidence Intervals	
Slope	5.783 to 6.242
Y-intercept	-0.08641 to 0.1384
Goodness of Fit	
r^2	0.9996

c



Variables	
Slope	4.591 ± 0.1512
Y-intercept	0.0004000 ± 0.07409
X-intercept	-0.00008714
1/slope	0.2178
95% Confidence Intervals	
Slope	4.109 to 5.072
Y-intercept	-0.2353 to 0.2361
Goodness of Fit	
r^2	0.9968

d



Variables	
Slope	0.01068 ± 0.0004044
Y-intercept	0.06484 ± 0.02913
X-intercept	-6.072
1/slope	93.65
95% Confidence Intervals	
Slope	0.008938 to 0.01242
Y-intercept	-0.06051 to 0.1902
Goodness of Fit	
r^2	0.9971

Figure A.1. Calibration curves for the conversion of absorbency readings to measurements of enzyme activity.

(a) N-acetylglucosamine, (b) glucose, (c) xylose and (d) p-nitroaniline.

Appendix B

Additional techniques and worked examples

B.1 Production and secretion of lectins by T. harzianum

The method of Neethling and Nevalainen (1996) was used to induce lectin production by isolates of *T. harzianum* (Th1 and Th2). Spore suspensions (1ml volumes) of 1.0 x 10^7 spores/ml were added to 100ml of the following liquid medium in 250ml conical flasks. The liquid medium (Kellens and Peumans, 1990) contained (l^{-1}): 2.0g L-asparagine, 15.0g D-glucose, 1.4g KH_2PO_4, 1.0g $MgSO_4.7H_2O$, 1.0 ml of a x1000 mineral stock (500 ml^{-1}): 4.4mg $ZnSO_4.7H_2O$, 1.0mg $FeSO_4.7H_2O$, 86µg $MnCl_2.4H_2O$, 79µg $CuSO_4.5H_2O$, 53µg $Na_2MoO_4.2H_2O$ (pH5.2) and 10µl per 100ml of medium thiamin hydrochloride (10mg/ml).

Flasks were incubated at 25°C on a rotary shaker at 150 rpm in the dark. Three replicate flasks were harvested after 3, 6, 9 and 13 days. Mycelia were collected in muslin, freeze-dried overnight and then ground and stored at -20°C until extraction. Filtrates were divided into aliquots, flash frozen in liquid nitrogen and stored at -20°C. Prior to haemagglutination assays freeze-dried mycelia were extracted using the method described by Neethling and Nevalainen (1996). Mycelium was suspended in 10 ml phosphate buffered saline (PBS) [pH 7.2] and shaken at 100 rpm at 4°C for 30 min. The sample was clarified by centrifugation at 2000 x *g* for 10 min. The supernatant was removed and the sediment extraction repeated twice, the combined supernatants were thought to contain cytosolic and cell envelope components (CM fraction).

Haemagglutination assays were performed using group AB human red blood cells (RBC) in 2% (v/v) suspensions. Equal volumes (50µl) of sample and RBC were added to a well in a round-bottomed microtitre plate. The plate was incubated at 37°C for 30 min and then at 4°C overnight. Assessment was then made visually using the following scale: full haemagglutination reaction (++) seen as a carpet of RBC in the bottom of the well; partial reaction (+) was recorded for slightly less coverage than the full reaction; negative reaction (-) occurred when a small button of cells collected in the bottom of the well.

Both untreated or neuraminidase (ex- *Vibrio cholerae*, Sigma) treated RBC were used for the haemagglutination assay. This increased the range of surface sugars available for lectin binding, since neuraminidase treatment removed terminal sialic acid residues on the cell surface. RBC were resuspended in 50mM sodium acetate (pH5.5) and then incubated at 37°C with neuraminidase at 60mU ml^{-1} for 40 min, then washed and resuspended in 10mM PBS (pH7.4).

All *T. harzianum* isolates tested exhibited haemagglutination activity in filtrates and CM fraction (Table B.1). The neuraminidase treated RBC demonstrated more full haemagglutination reactions than did untreated RBC. Genotype differences were more obvious with untreated RBC as only the filtrates from Th2 exhibited agglutination activity, however this was partial. Comparing all the reactions the aggressive *T. harzianum* exhibited more full haemagglutination reactions than did the non-aggressive *T. harzianum.*

Sugar inhibition assays were performed in an attempt to determine the sugar specificity of the haemagglutination activity (Sharon and Lis 1989). Lectins bind to carbohydrates via multivalent binding sites and can therefore be classified into monosaccharide specificity groups by determination of the most effective monosaccharide inhibitor of haemagglutination. The binding of monsaccharides to the lectin binding sites prevents RBC attachment resulting in a negative haemagglutination reaction. The low molecular weight sugars (haptens) described by Neethling and Nevalainen (1996) were used at 100 mM concentration and were as follows: N-acetylgalactosamine, N-acetylglucosamine, D-galactose, D-mannose, L-fucose, D-lactose, D-melibiose, fetuin, methyl-β-D-glucopyranoside, methyl-α-D-mannoside and sialic acid. Lectin samples and sugar solutions (1:1 v/v) were incubated at 37°C for 1 hour and subsequently 50 µl was added to a microtitre well with 50 µl of RBC for a haemagglutination assay. A negative reaction was indicative of inhibition of agglutination activity by the binding of a specific hapten.

Table B.1. Detection of secreted and cell-associated lectins produced by isolates of *T. harzianum*.

T. harzianum isolate	Culture Age (days)	Culture Filtrate*		CM Fraction†	
		Untreated RBC[a]	Treated RBC	Untreated RBC	Treated RBC
Th1(c)	3	-	++	++	++
	6	-		+	++
	9	-	++	+	++
	13	-	+	-	++
TD15	3	-	+	++	++
	6	-	+	+	++
	9	-	+	-	+
	13	-	+	-	++
T7	3	+	++	++	++
	6	+	+	++	++
	9	+	+	++	++
	13	+	+	+	++
T32	3	+	++	++	++
	6	+	++	+	++
	9	+	++	-	++
	13	+	++	-	++

All haemagglutination assays were performed using group AB RBC. * Culture filtrates contained possible secreted lectins. † CM fraction should contain cytosolic and cell envelope components. [a] Untreated RBC were suspended in PBS, while treated RBC were first incubated with neuraminidase at 60mU ml^{-1} for 40 min at 37°C to remove terminal sialic acid residues.
T. harzianum genotypes: Th1: Th1(c) and TD15; Th2: T7 and T32.

Table B.2. Determination of carbohydrate specificity of secreted and cell-associated lectins produced by isolates of *T. harzianum*.

T. harzianum isolate/age (days)	Extract Fraction	Sugar haptens				
		D-gal	D-lac	D-meli	fetuin	sialic acid
Th1(c) 3d	F				+	
Th1(c) 9d	F				+	
TD15 3d	F				+	
TD15 6d	F	+			+	+
T7 6d	F		+			
T7 13d	F				+	+
T32 3d	F			+	+	
T32 6d	F			+	+	
T32 9d	F				+	
T32 13d	F				+	
Th1(c) 3d	CM				+	
T32 3d	CM				+	

+ denotes inhibition of haemagglutination.

* Secreted lectins present in the culture filtrates were termed 'F'. Cell-associated lectin samples were termed 'CM'. Haemagglutination assays performed after sugar inhibition used neuraminidase-treated RBC for secreted lectins and untreated RBC for cell-associated lectins. Group AB was used throughout. *T. harzianum* genotypes described in legend for Table B.1.

The use of neuraminidase-treated RBC revealed more lectin activity than untreated blood (Table B.1) and this suggested a role for sialic acid in the agglutination reaction. Paradoxically the addition of sialic acid failed to inhibit most lectin agglutination (Table B.2). Lectins produced by isolates of *T. harzianum* appeared to be inhibited by fetuin, with the exception of a lectin produced by isolate T7 (Th2) after 6 days incubation. Fetuin is a glycoprotein of fetal serum of which 22.5% is carbohydrate and 74% protein (Spiro 1960). The carbohydrate fraction comprises several sugars of which galactose, glucosamine and sialic acids represent the highest proportions. Therefore it would appear that the majority *T. harzianum* lectin activity produced under these conditions were specific for sialic acid as the terminal residue in an oligosaccharide. Spiro (1960) confirmed that sialic acid was present as terminal residues on the carboxyl groups. Some lectin activity was inhibited by D-galactose and sialic acid as well as fetuin suggesting that lectin activity also recognised the component sugars. Notably, some Th2 lectins were specific for D-lactose and D-melibiose thus indicating a possible difference between *T. harzianum* genotypes. It may be valuable to assess the lectins produced by all *T. harzianum* genotypes on exposure to *A. bisporus* cell walls.

B.2. Detection of depolymerase activity by T. harzianum on sterile compost in the absence of A. bisporus.

Trichoderma harzianum was allowed to colonise sterile mushroom compost and depolymerases were extracted in the manner described in Chapter Five (section 5.1.4). Two separate experiments were performed using different batches of compost during which, depolymerases were extracted after 1, 2 and 3 weeks of incubation. Depolymerase activity was determined by Nelson-Somogyi sugar determination method (Nelson 1944; Somogyi 1952).

Differences in enzyme activities were detected between *T. harzianum* genotypes after the varying periods of incubation (Fig B.1-B.4). Since there was no apparent pattern to the differences with time it is likely that contrast reflects the level of growth. Quantification of *T. harzianum* growth in sterile compost in conjunction with depolymerase investigations may alleviate such discrepancies.

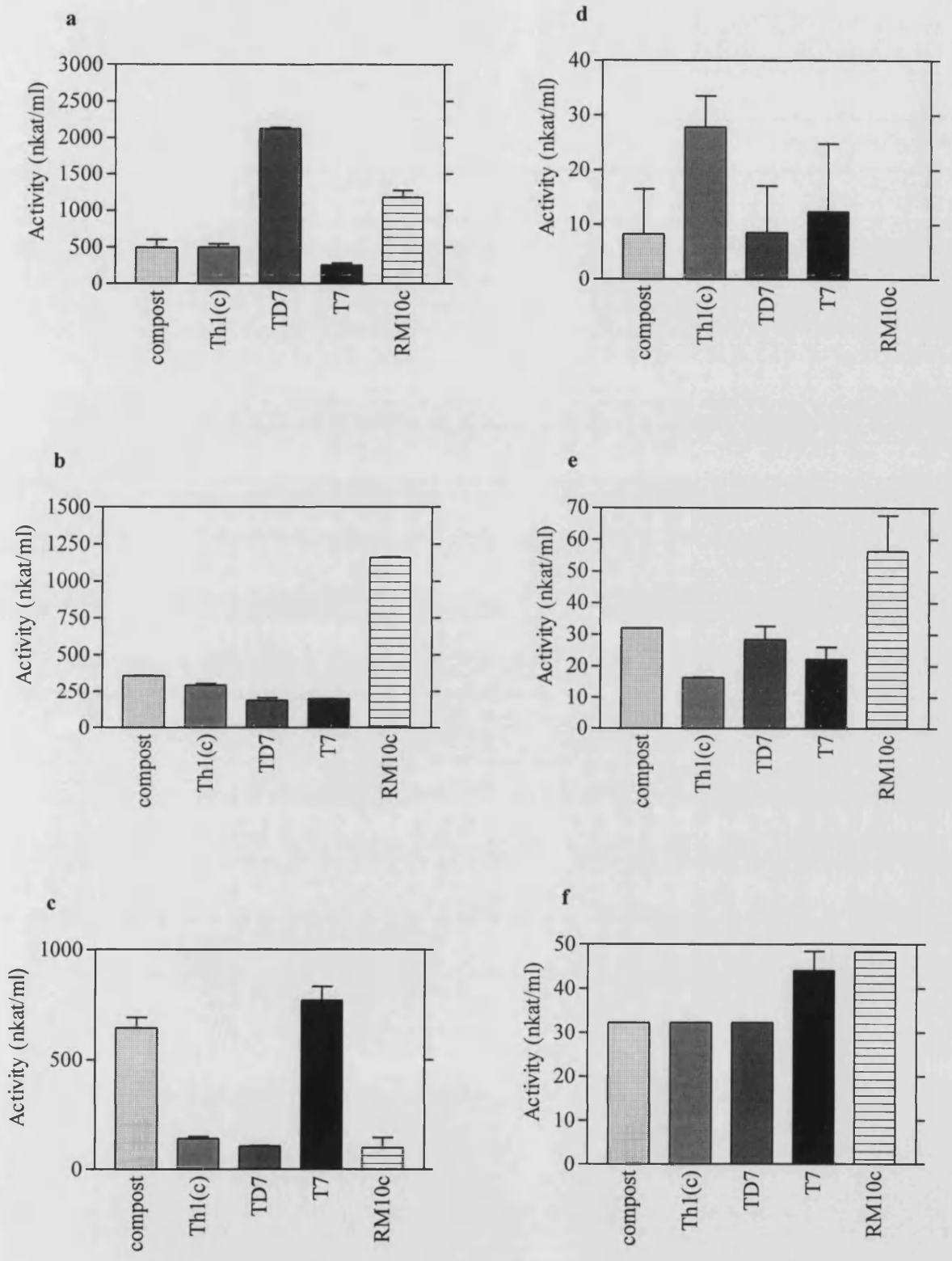


Figure B.1. Detection of *T. harzianum* depolymerase activity from sterile compost (trial 1).

(a) to (c) Xylanase activity and (e) to (f) cellulase activity: (a) and (d) 1 week, (b) and (e) 2 weeks and (c) and (f) 3 weeks incubation. *T. harzianum* isolates: Th1(c) (Th1), TD7 (Th3), T7 and Th2A (Th2) and RM10c (Th4).

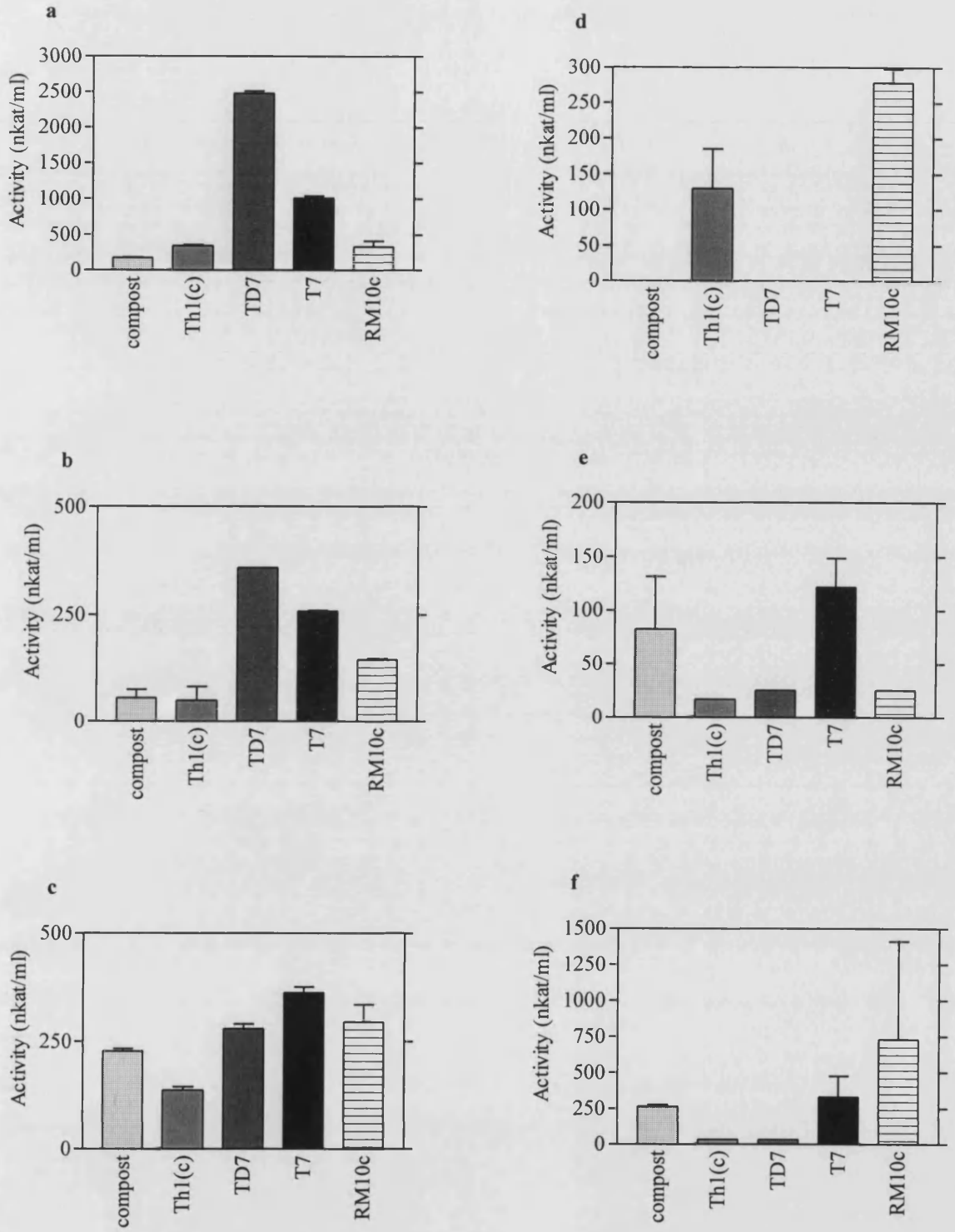


Figure B.2. Detection of *T. harzianum* depolymerase activity from sterile compost (trial 1).

(a) to (c) laminarinase activity and (e) to (f) chitinase activity: (a) and (d) 1 week, (b) and (e) 2 weeks and (c) and (f) 3 weeks incubation. *T. harzianum* isolates see Fig B.1 legend.

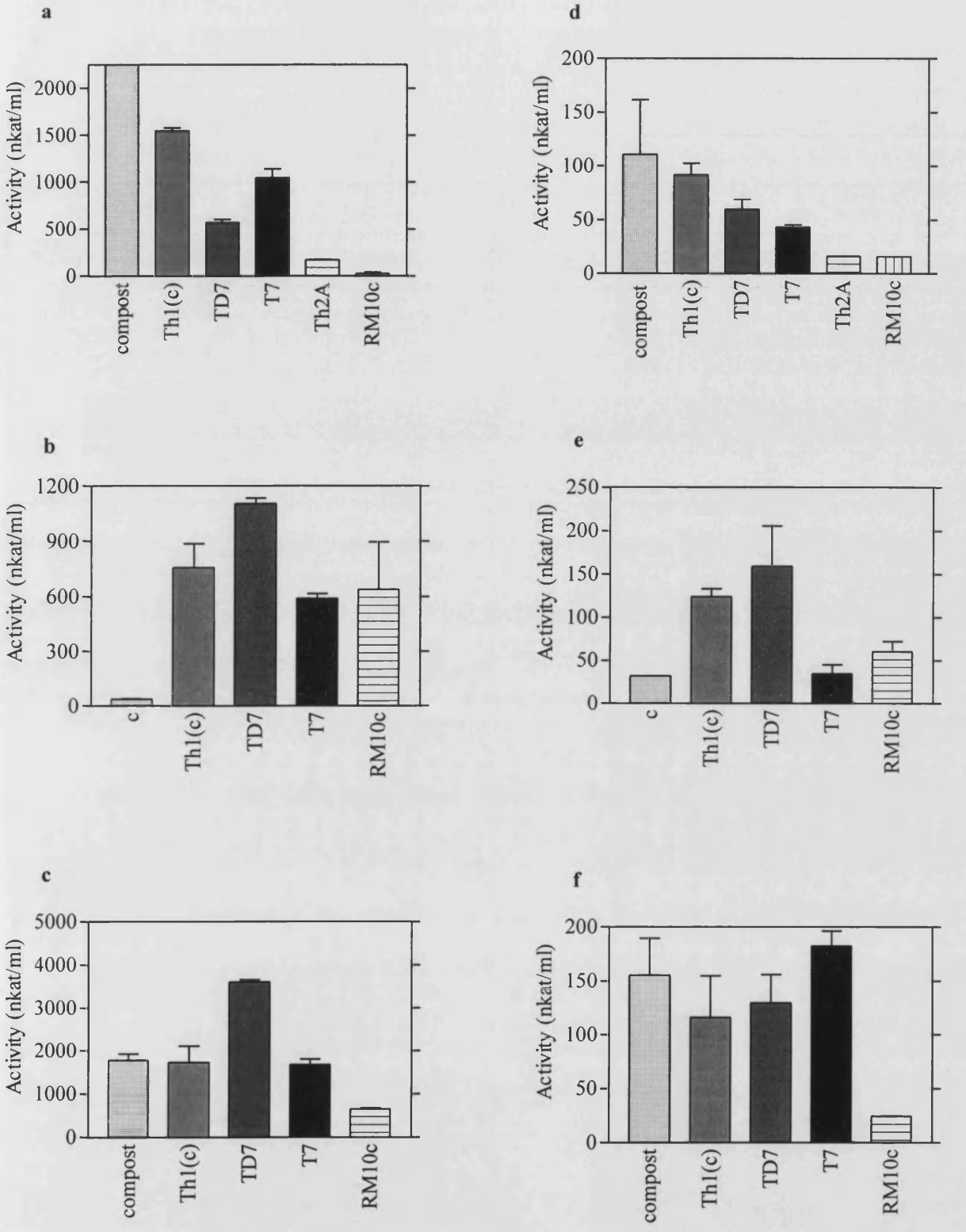


Figure B.3. Detection of *T. harzianum* depolymerase activity from sterile compost (trial 2). (a) to (c) Xylanase activity and (e) to (f) cellulase activity: (a) and (d) 1 week, (b) and (e) 2 weeks and (c) and (f) 3 weeks incubation. For *T. harzianum* isolates see Figure B.1 legend.

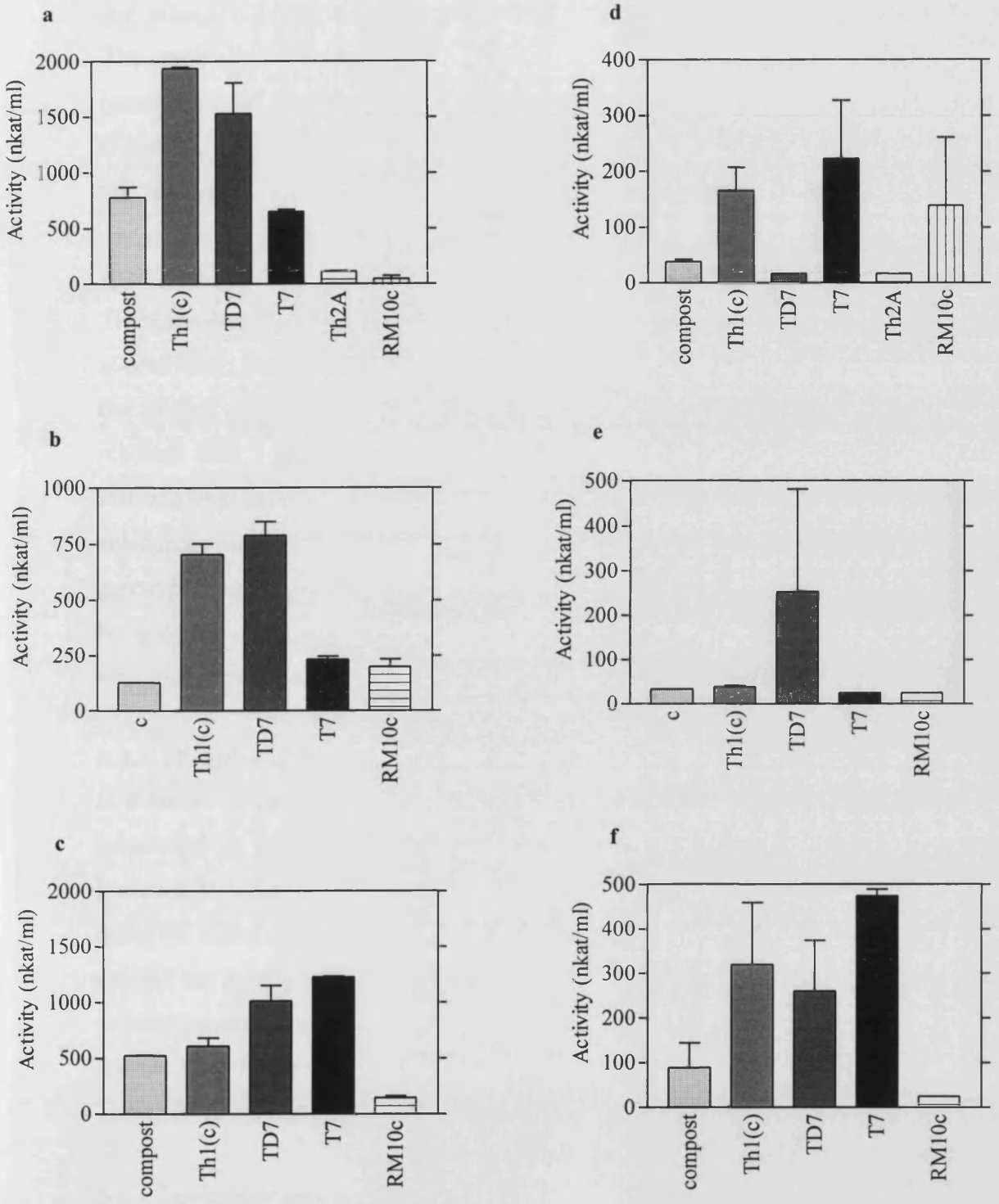


Figure B.4. Detection of *T. harzianum* depolymerase activity from sterile compost (trial 2). (a) to (c) laminarinase activity and (e) to (f) chitinase activity: (a) and (d) 1 week, (b) and (e) 2 weeks and (c) and (f) 3 weeks incubation. For *T. harzianum* isolates see Figure B.1 legend.

B.3. Worked examples of statistical analyses.

The statistical analyses applied to the data have been described in Chapter Three (section 3.1.1). The distribution of the data was first determined using the normality test (Prism® Graph Pad software) to ensure that the correct statistical analysis was used. In this section some examples of the process of statistical analysis are given.

B.3.1 Production of non-volatile substances by T. harzianum

Three nutrient agar media were used to provide a range of nutrient concentrations and accessibility; in addition growth measurements were recorded over several days. Due to the amount of statistical analysis only one example may be shown here. The diameter readings after 7 days incubation on DWA are shown here after unpaired, two-tailed t-test analysis between *A. bisporus* control and *A. bisporus* exposed to non-volatile substances produced by *T. harzianum* (Fig B.5). To compare the effects of *T. harzianum* genotypes on *A. bisporus* growth the data was corrected by using the differences between 'control' and 'treatment' diameters. The corrected data was subjected to one-way analysis of variance (ANOVA) and an example of this is shown (Fig B.6).

B.3.2. The effect of T. harzianum on the growth of A. bisporus in compost.

In a model of the industrial green mould problem, *T. harzianum* and *A. bisporus* were introduced to mushroom compost in a screw-capped plastic tube. The effect of the presence of *T. harzianum* towards the growth of *A. bisporus* was followed and assessed using the visual scale described in Chapter Three (section 3.1.2). The normality test was applied to determine the distribution of the data (Table B.3). The normality test determined the data as non-parametric and therefore the Friedman test was performed followed by Dunn's multiple comparison test. The analysis for Table 3.9 (Chapter Three) is shown here as an example (Fig B.7).

B.3.3. Saprophytic growth of T. harzianum in compost.

In Chapter Five (section 5.1.1(ii)) the quantification of *T. harzianum* saprophytic growth in compost and the statistical analyses used to compare subsequent *T. harzianum* biomass were described. An example of the analysis is shown using data from a replicate experiment (Fig B.8). The cfu counts were first converted to $\log_{10}$ and then subjected to one-way ANOVA (Table B.4). As seen in Chapter Five (section 5.1.1(ii)) genotypes 2, 3 and 4 exhibited significantly higher saprophytic growth than did Th1.

Unpaired t test	
P value	0.0006
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.520 df=6
How big is the difference?	
Mean ± SEM of column A	12.55 ± 0.09354 N=5
Mean ± SEM of column B	14.33 ± 0.3333 N=3
Difference between means	-1.783 ± 0.2735
95% confidence interval	1.114 to 2.453
R squared	0.8763
F test to compare variances	
F,DFn, Dfd	7.619, 2, 4
P value	0.0432
P value summary	*
Are variances significantly different?	Yes

Table Analyzed	Data Table-7 Columns A and C
Unpaired t test	
P value	0.0346
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.721 df=6
How big is the difference?	
Mean ± SEM of column A	12.55 ± 0.09354 N=5
Mean ± SEM of column C	11.50 ± 0.5000 N=3
Difference between means	1.050 ± 0.3859
95% confidence interval	-1.994 to -0.1058
R squared	0.5524
F test to compare variances	
F,DFn, Dfd	17.14, 2, 4
P value	0.0109
P value summary	*
Are variances significantly different?	Yes

Figure B.5. The effect of non-volatile substances produced by *T. harzianum* on the growth of *A. bisporus*. Unpaired, two-tailed t-test analysis. Columns: A, *A. bisporus* control; B, effect of Th1(c) (Th1); C, effect of T7 (Th2); D, effect of Th2F (Th2).

growth on DWA after 7 days incubation.

Table Analyzed	Data Table-7 Columns A and D
Unpaired t test	
P value	0.6374
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4899 df=8
How big is the difference?	
Mean ± SEM of column A	12.55 ± 0.09354 N=5
Mean ± SEM of column D	12.40 ± 0.2915 N=5
Difference between means	0.1500 ± 0.3062
95% confidence interval	-0.8561 to 0.5561
R squared	0.02913
F test to compare variances	
F,DFn, Dfd	9.714, 4, 4
P value	0.0245
P value summary	*
Are variances significantly different?	Yes

Parameter	Value	Data Set-B	Data Set-C	Data Set-D
Table Analyzed				
Data Table-7				
One-way analysis of variance				
P value	0.0040			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	11.89			
R squared	0.7483			
ANOVA Table	SS	df	MS	
Treatment (between columns)	12.47	2	6.235	
Residual (within columns)	4.195	8	0.5243	
Total	16.67	10		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
a12/th1 diff vs a12/t7 diff	-2.833	4.792	P < 0.01	-4.616 to -1.050
a12/th1 diff vs a12/th2f diff	-1.813	3.429	P < 0.05	-3.408 to -0.2185
a12/t7 diff vs a12/th2f diff	1.020	1.929	P > 0.05	-0.5748 to 2.615

Figure B.6. The effect of non-volatile substances produced by *T. harzianum* on the growth of *A. bisporus*. One-way ANOVA and Bonferroni's post test. *T. harzianum* strains: Th1(c) (Th1); T7, T32 and Th2F (Th2).

single tube 11/6/97	a12	a12/th1	a12/td15	a12/t7	a12/t32
Number of values	10	10	10	10	10
Minimum	2.000	2.000	2.000	0.0000	0.0000
25% Percentile	3.000	3.000	2.500	0.0000	0.0000
Median	3.000	3.000	3.000	0.0000	0.0000
75% Percentile	3.000	3.000	3.000	0.0000	0.0000
Maximum	3.000	3.000	3.000	0.0000	1.000
Mean	2.800	2.800	2.700	0.0000	0.1000
Std. Deviation	0.4216	0.4216	0.4830	0.0000	0.3162
Std. Error	0.1333	0.1333	0.1528	0.0000	0.1000
Lower 95% CI	2.498	2.498	2.354	0.0000	-0.1262
Upper 95% CI	3.102	3.102	3.046	0.0000	0.3262
Normality Test					
KS distance	0.4824	0.4824	0.4327	0.5000	0.5241
P value	0.0191	0.0191	0.0473	0.0135	0.0082
Passed normality test (*=0.05)?	No	No	No	No	No
P value summary	*	*	*	*	**

Table B.3. Column statistics and normality test for data representative of *A. bisporus* growth in the presence of *T. harzianum*. For *T. harzianum* genotypes see Fig B.6 legend.

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Data Table-1			
Friedman test			
P value	P<0.0001		
Exact or approximate P value?	Gaussian Approximation		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
Friedman statistic	28.64		
Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
th1 diff vs td15 diff	-1.000	P > 0.05	ns
th1 diff vs t7 diff	-21.00	P < 0.01	**
th1 diff vs t32 diff	-20.00	P < 0.01	**
td15 diff vs t7 diff	-20.00	P < 0.01	**
td15 diff vs t32 diff	-19.00	P < 0.01	**
t7 diff vs t32 diff	1.000	P > 0.05	ns

Parameter	Value	Data Set-B	Data Set-C
Table Analyzed			
Data Table-1			
Friedman test			
P value	P<0.0001		
Exact or approximate P value?	Gaussian Approximation		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	5		
Friedman statistic	36.24		
Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
a12 vs a12/th1	0.0000	P > 0.05	ns
a12 vs a12/td15	1.500	P > 0.05	ns
a12 vs a12/t7	26.00	P < 0.01	**
a12 vs a12/t32	25.00	P < 0.01	**
a12/th1 vs a12/td15	1.500	P > 0.05	ns
a12/th1 vs a12/t7	26.00	P < 0.01	**
a12/th1 vs a12/t32	25.00	P < 0.01	**
a12/td15 vs a12/t7	24.50	P < 0.01	**
a12/td15 vs a12/t32	23.50	P < 0.01	**
a12/t7 vs a12/t32	-1.000	P > 0.05	ns

Figure B.7. The effect of *T. harzianum* on the growth of *A. bisporus* in non-sterile mushroom compost. Friedman test followed by Dunn's multiple comparison test. For *T. harzianum* genotypes see Fig B.6 legend.

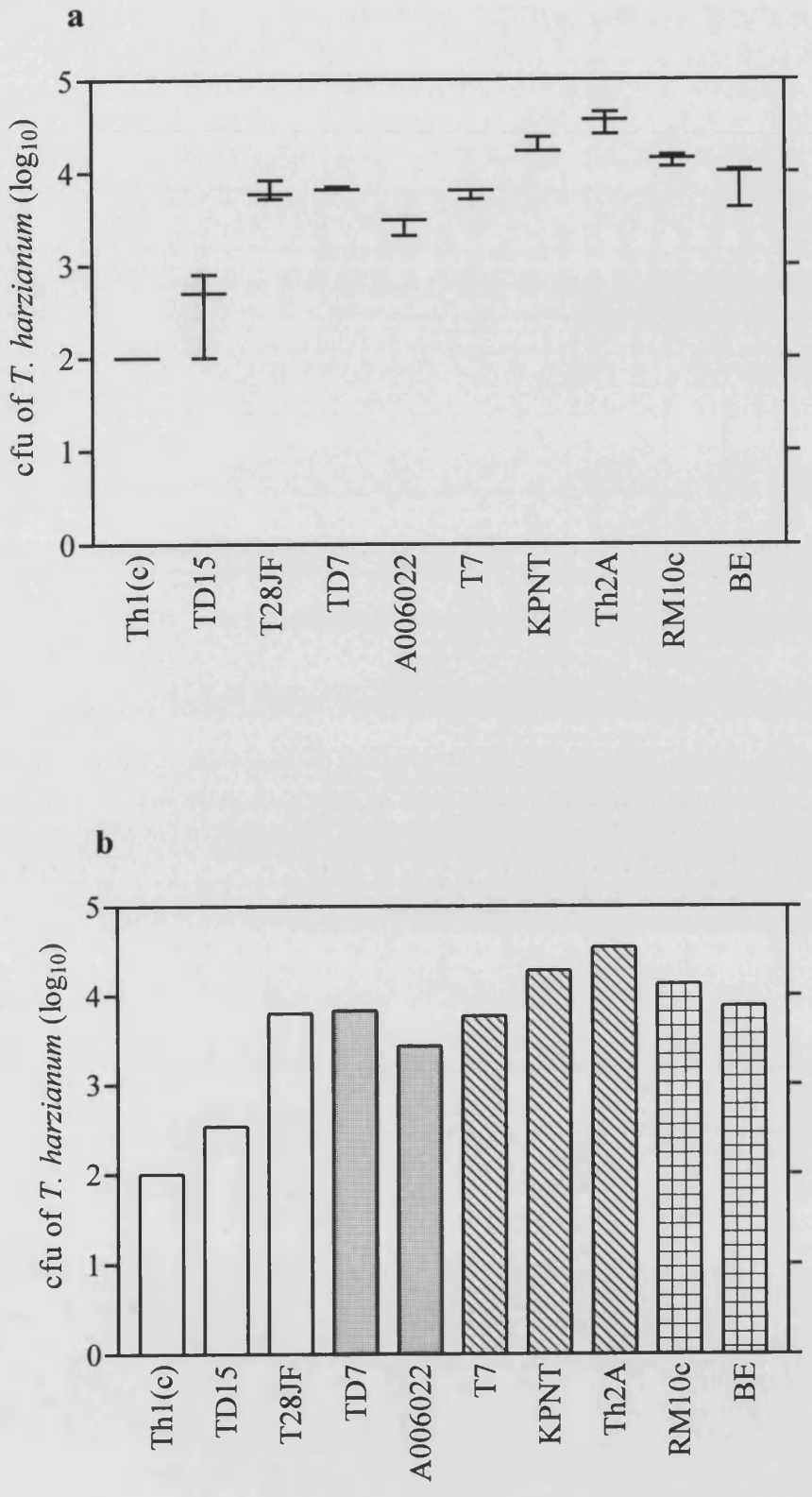


Figure B.8. Saprophytic growth of *T. harzianum* in mushroom compost.

(a) Box plot with whiskers. (b) Bar chart of $\log_{10}$ of cfu.

Th1: Th1(c), TD15, T28JF

Th2: T7, KPNT, Th2A

Th3: TD7, A006022

Th4: RM10c, BE

Parameter	Value	Data Set-B	Data Set-C	Data Set-D
Table Analyzed				
Data Table-3				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	10			
F	44.81			
R squared	0.9550			
ANOVA Table	SS	df	MS	
Treatment (between columns)	13.96	9	1.551	
Residual (within columns)	0.6577	19	0.03462	
Total	14.62	28		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
Th1(c) vs TD15	-0.5343	3.146	P > 0.05	-1.186 to 0.1174
Th1(c) vs T28JF	-1.802	10.61	P < 0.001	-2.454 to -1.150
Th1(c) vs TD7	-1.830	10.77	P < 0.001	-2.482 to -1.178
Th1(c) vs A006022	-1.433	8.439	P < 0.001	-2.085 to -0.7816
Th1(c) vs T7	-1.774	10.44	P < 0.001	-2.426 to -1.122
Th1(c) vs KPNT	-2.280	13.42	P < 0.001	-2.932 to -1.628
Th1(c) vs Th2A	-2.543	14.97	P < 0.001	-3.194 to -1.891
Th1(c) vs RM10c	-2.133	12.56	P < 0.001	-2.785 to -1.482
Th1(c) vs BE	-1.887	11.11	P < 0.001	-2.538 to -1.235
TD15 vs T28JF	-1.268	8.345	P < 0.001	-1.851 to -0.6847
TD15 vs TD7	-1.296	8.529	P < 0.001	-1.879 to -0.7127
TD15 vs A006022	-0.8990	5.918	P < 0.001	-1.482 to -0.3161
TD15 vs T7	-1.240	8.160	P < 0.001	-1.823 to -0.6567
TD15 vs KPNT	-1.746	11.49	P < 0.001	-2.329 to -1.163
TD15 vs Th2A	-2.008	13.22	P < 0.001	-2.591 to -1.425
TD15 vs RM10c	-1.599	10.53	P < 0.001	-2.182 to -1.016
TD15 vs BE	-1.352	8.902	P < 0.001	-1.935 to -0.7694
T28JF vs TD7	-0.02800	0.1843	P > 0.05	-0.6109 to 0.5549
T28JF vs A006022	0.3687	2.427	P > 0.05	-0.2143 to 0.9516
T28JF vs T7	0.02800	0.1843	P > 0.05	-0.5549 to 0.6109
T28JF vs KPNT	-0.4780	3.147	P > 0.05	-1.061 to 0.1049
T28JF vs Th2A	-0.7407	4.876	P < 0.01	-1.324 to -0.1577
T28JF vs RM10c	-0.3313	2.181	P > 0.05	-0.9143 to 0.2516
T28JF vs BE	-0.08467	0.5573	P > 0.05	-0.6676 to 0.4983
TD7 vs A006022	0.3967	2.611	P > 0.05	-0.1863 to 0.9796
TD7 vs T7	0.05600	0.3686	P > 0.05	-0.5269 to 0.6389
TD7 vs KPNT	-0.4500	2.962	P > 0.05	-1.033 to 0.1329
TD7 vs Th2A	-0.7127	4.691	P < 0.01	-1.296 to -0.1297
TD7 vs RM10c	-0.3033	1.997	P > 0.05	-0.8863 to 0.2796
TD7 vs BE	-0.05667	0.3730	P > 0.05	-0.6396 to 0.5263
A006022 vs T7	-0.3407	2.242	P > 0.05	-0.9236 to 0.2423
A006022 vs KPNT	-0.8467	5.573	P < 0.01	-1.430 to -0.2637
A006022 vs Th2A	-1.109	7.302	P < 0.001	-1.692 to -0.5264
A006022 vs RM10c	-0.7000	4.608	P < 0.01	-1.283 to -0.1171
A006022 vs BE	-0.4533	2.984	P > 0.05	-1.036 to 0.1296
T7 vs KPNT	-0.5060	3.331	P > 0.05	-1.089 to 0.07693
T7 vs Th2A	-0.7687	5.060	P < 0.01	-1.352 to -0.1857
T7 vs RM10c	-0.3593	2.365	P > 0.05	-0.9423 to 0.2236
T7 vs BE	-0.1127	0.7416	P > 0.05	-0.6956 to 0.4703
KPNT vs Th2A	-0.2627	1.729	P > 0.05	-0.8456 to 0.3203
KPNT vs RM10c	0.1467	0.9655	P > 0.05	-0.4363 to 0.7296
KPNT vs BE	0.3933	2.589	P > 0.05	-0.1896 to 0.9763
Th2A vs RM10c	0.4093	2.695	P > 0.05	-0.1736 to 0.9923
Th2A vs BE	0.6560	4.318	P < 0.05	0.07307 to 1.239
RM10c vs BE	0.2467	1.624	P > 0.05	-0.3363 to 0.8296

Table B.4. Comparison saprophytic growth of *T. harzianum* in compost.

One-way ANOVA followed by Bonferroni's multiple comparison test. For *T. harzianum* genotypes see Figure B.8 legend.